

Alpha₁-Antitrypsin and the Liver

Clinical and Biosynthetic Studies

Joyce Carlson



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ERRATA

Page 31, line 17 continous should be continuous.

Page 32, ref. 18 should be: Fagerhol MK, Quantitative Studies on the inherited variants of serum alpha-1-antitrypsin, Scand J Clin Lab Invest, 1969;23:97-103.

Page 37, column II, line 41 steatosis has been omitted.

Page 38, column I, bottom line should be **** $p < 0.001$.

Page 40, column II, line 8 boned should be bone.

Page 41, column II, line 2 ration should be ratio.

Page 42, column I, line 10 hepatoma²³ should be hepatoma^{2,3}.

Page 48, Abbreviations line 2 aspartateamino should be aspartate amino.

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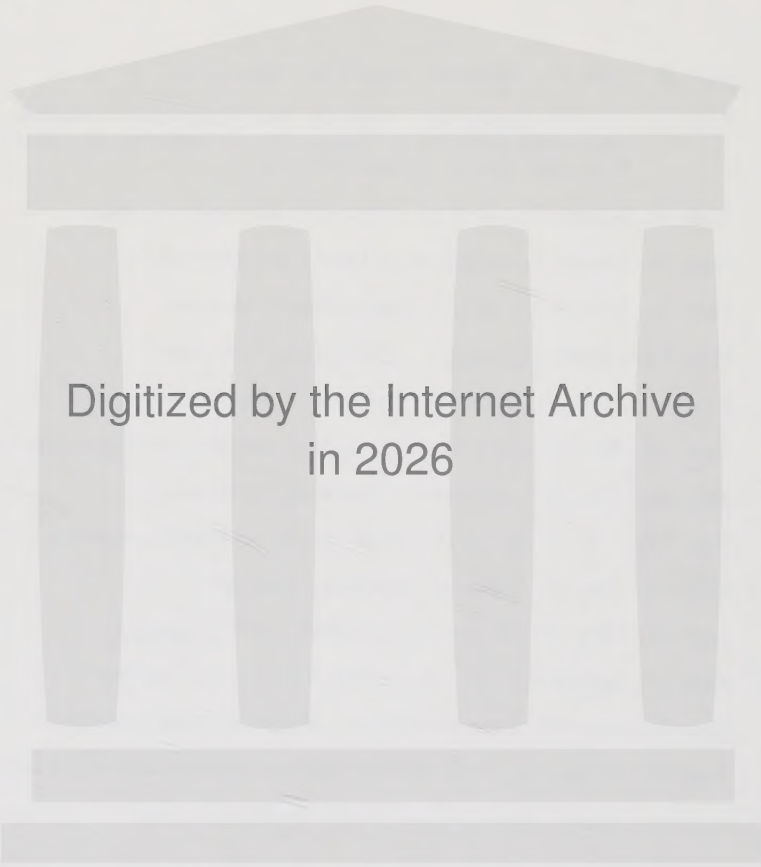
Page 52, line 20 D) cirrhosis should be D) cirrhosis.

Page 57, bottom 2 lines X method should be χ^2 method.

Page 58, line 13 hemachromatosis should be hemochromatosis.

Page 69, column II, line 12 from bottom content should be context.

Page 74, line 3 pepstatin 10 ml/l should be mg/ml.



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ALPHA₁-ANTITRYPSIN AND THE LIVER
Clinical and Biosynthetic Studies

AKADEMISK AVHANDLING

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Abstract Plasma alpha ₁ -antitrypsin (α ₁ -AT) concentrations increase selectively with increasing inflammation but not with cirrhosis, fibrosis or steatosis in concomitant liver biopsy specimens. α ₁ -AT contributes to an acute phase reactant pattern which may aid in the clinical evaluation of the severity of liver disease.		
Using a monoclonal antibody specific for Pi Z α₁-AT, an overrepresentation of male heterozygotes for the Pi Z deficiency was found among 861 patients with liver disease. Cryptogenic liver disease was nearly 5 times more common in heterozygotes than in sex- and age matched controls from the same study population. Malignant hepatoma was present in 6 of 64 heterozygotes compared to 1 of 128 controls (p<0.01) and 13 of all 793 non Pi Z patients in the study population (p<0.001). Carriers of the Pi Z deficiency allele accumulate globular inclusions of α₁-AT in the RER of hepatocytes. It appears that lively biosynthesis of Pi Z α₁-AT and the resulting globular inclusions contribute to the development of liver disease in the hetero- as well as the homozygous Pi Z deficiency state. For this reason, the biosynthesis and secretion of α₁-AT were studied in isolated rat hepatocytes and in a human hepatoma cell line.		
α₁-AT is translated with an NH₂-terminal signal peptide of 24 mainly hydrophobic amino acids which is removed after penetration of the endoplasmic reticulum membrane. Three core-oligosaccharides are added cotranslationally, further modified in the SER and Golgi, and secreted in the terminally glycosylated state after about 45 min. Secretion is delayed but not prevented by tunicamycin, a substance which inhibits core-glycosylation. Synthesis and secretion are similar in the two cell systems, but globular inclusions of α₁-AT, similar but not identical to Pi Z globules, are formed in the hepatoma cells. These cells secrete abnormally glycosylated α₁-AT with increased carbohydrate branching and decreased sialic acid content.		
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Alpha₁-Antitrypsin and the Liver

Clinical and Biosynthetic Studies

by

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1984

This dissertation is based on the following publications which will be referred to by Roman numerals I-V:

- I. Carlson J, Eriksson S. α_1 -Antitrypsin and other acute phase reactants in liver disease. Acta Med Scand 1980; 207:79-83.
- II. Carlson J, Eriksson S, Hägerstrand I. Intra- and extracellular α_1 -antitrypsin in liver disease with special reference to Pi-phenotype. J Clin Pathol 1981; 34:1020-5.
- III. Carlson J, Eriksson S. Chronic "cryptogenic" liver disease and malignant hepatoma in intermediate α_1 -antitrypsin deficiency identified by a Pi Z-specific monoclonal antibody. Submitted.
- IV. Carlson J, Stenflo J. The biosynthesis of rat α_1 -antitrypsin. J Biol Chem 1982; 257:12987-994.
- V. Carlson J, Eriksson S, Alm R, Kjellström T. The biosynthesis of abnormally glycosylated α_1 -antitrypsin in a human hepatoma cell line. In press, Hepatology.

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ABBREVIATIONS

α_1 -AT	= α_1 -Antitrypsin
ELISA	= Enzyme-linked immunosorbent assay
endo-H	= Endo-N- β -acetylglucosaminidase H
IEF	= Isoelectric focusing
PAS	= Periodic acid-Schiff
Pi	= Protease inhibitor
RER	= Rough endoplasmic reticulum
SER	= Smooth endoplasmic reticulum

INTRODUCTION

Alpha₁-antitrypsin (α_1 -AT), the major serine protease inhibitor in human plasma, is a glycoprotein synthesized in the liver. Clinical interest was first focused on α_1 -AT in 1963 when an hereditary deficiency was discovered and found to be associated with pulmonary emphysema (1). In 1969 an American family with childhood cirrhosis was also reported to have the deficiency (2) and periodic acid-Schiff (PAS)-positive inclusion bodies, shown to consist of aggregated α_1 -AT were later found in the hepatocytes of such patients (3). Since then numerous reports have substantiated an association between homozygous α_1 -AT deficiency and chronic liver disease in adults (4, 5). One recent report from England has found a clear overrepresentation of patients with heterozygous α_1 -AT deficiency among subjects with so called cryptogenic liver cirrhosis and non-B chronic active hepatitis (6).

The association of α_1 -AT deficiency with liver disease suggests that this protein may play an essential role in liver disease per se and that intracellular accumulation of aggregated α_1 -AT may have a toxic effect on hepatocytes. To gain further insight into these problems we have used conventional methods to study intra- and extracellular α_1 -AT in patients with various liver diseases and to determine the phenotypic distribution among such patients. Carriers of the Pi Z allele have also been identified using a monoclonal antibody and the panorama of liver disease among these subjects has been compared to that among patients lacking the Z gene. To gain further insight into the process of secretion and the "secretory defect" in α_1 -AT deficiency, the biosynthesis of α_1 -AT has been studied in isolated rat hepatocytes and in a human hepatoma cell line known to accumulate α_1 -AT globular inclusions (7).

THE BIOLOGICAL SIGNIFICANCE OF ALPHA₁-ANTITRYPSIN

The Protein and its Genetic Variants

The major antiprotease activity in human plasma was located in the alpha₁-electrophoretic globulin fraction in 1955 (8), and a glycoprotein responsible for this activity was identified in 1962 and called α_1 -AT (9). Shortly thereafter, the absence of the typical α_1 -AT band was discovered in the electrophoretic patterns of several patients with pulmonary emphysema (1). A very weak protein band with slightly retarded mobility in the alpha₁₋₂ region was detected and analysis by crossed immunoelectrophoresis after starch gel electrophoresis soon demonstrated the presence of several genetic α_1 -AT variants comprising the protease inhibitor (Pi) system (10).

Phenotyping is currently performed on polyacrylamide gel isoelectric focusing (IEF) and at least 30 variant alleles have been identified and are designated by letters of the alphabet according to their mobilities in this system (11). The most common allele is called M (Medium mobility) and the slowest is Z. Only a few alleles result in reduced serum concentrations of the protein. Normal serum concentration in Pi M has been established to be 1.35 g/l (12) and serum levels in other phenotypes compared to this level are Pi --, 0% (13); Pi Z, 15% (14); and Pi S, 60% (15). The gene for α_1 -AT has been found at a single locus on chromosome 14 (16) and autosomal co-dominant inheritance has been established. Heterozygotes thus normally have reduced concentrations as follows: Pi M-, 50%; Pi MZ, 57%; Pi MS, 80%; Pi SZ, 37%; etc. The frequency of the Z allele in Scandinavia is 0.026 (17), the S allele 0.026 (18) and the Pi - allele is 0.001 (19). The Pi Z allele is probably more common in Scandinavia than in many other countries.

The complete amino acid sequence and the sites of carbohydrate

α_1 -ANTITRYPSIN - Schematic structure

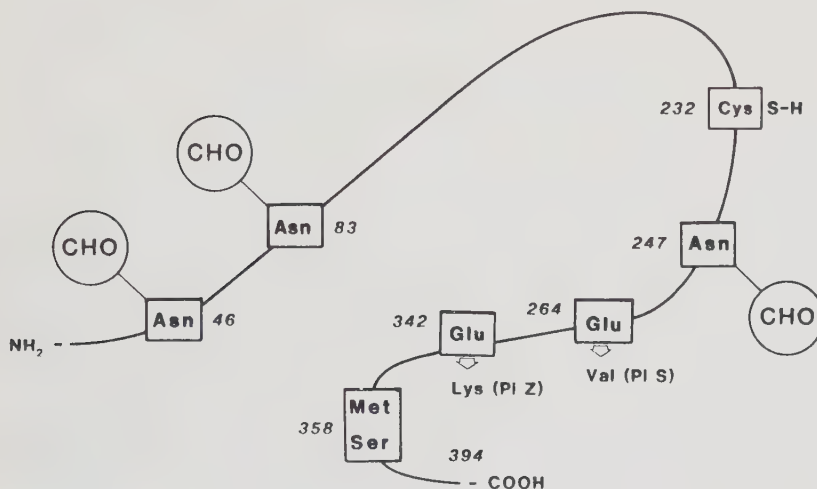


Figure 1. A schematic drawing showing the sites of carbohydrate attachments, the only cysteine residue, mutation sites and the active site of human α_1 -AT. No resemblance to the tertiary structure is intended.

attachment of human α_1 -AT are now known (20). Some features are summarized in Figure 1. The microheterogeneity normally seen on IEF can be partially explained by variations in the carbohydrate side chains in which the usual biantennary chains may be replaced in position 83, and to lesser degrees in positions 46 and 247 by triantennary oligosaccharides (21). Extra bands may be explained by a subpopulation lacking an N-terminal pentapeptide but with normal carbohydrate (personal communication, Jan-Olof Jeppsson) possibly representing "used" protein. Differences in the IEF patterns are further explained by amino acid substitutions in the polypeptide chain. The most important of these are the single base mutations causing Pi S (264 Glu $\rightarrow$ Val) (22) and Pi ZZ (342 Glu $\rightarrow$ Lys) (23) associated with decreased plasma levels of the protein. No bands are found in the Pi -- phenotype and this phenotype is thought to be due to deletion of a gene locus (24). Low plasma levels of Pi Z α_1 -AT appear to be secondary to the in-

tracellular accumulation whereas the Pi S variant is thought to be labile resulting in intracellular degradation but has a normal plasma half-life of 6 days (25).

The Function of Alpha₁-Antitrypsin

α_1 -AT has been shown to effectively inhibit serine proteases by 1:1 complex formation (26) and methionine at position 358 is its reactive center, functioning as a target for the active sites of such proteases (27). Evidence suggests that dissociation of the carboxy-terminal 36-residue peptide does not occur under physiological conditions but may be demonstrated under more drastic chemical conditions after complex formation (28). Judging from the association rate constants, α_1 -AT inhibits in decreasing order of effectiveness: leucocyte elastase > chymotrypsin > cathepsin G > anionic trypsin > cationic trypsin > plasmin > thrombin (29). Under physiological conditions α_1 -AT's major activity is directed against the first three enzymes named. Because of its relatively small molecular size, α_1 -AT is present in the extracellular fluids as well as in plasma during inflammatory reactions. Proteases bound to α_1 -AT are transferred to alpha₂-macroglobulin which is confined to the blood plasma and subsequently eliminated from the circulation by the reticuloendothelial system and degraded (30).

The amino acid sequence of α_1 -AT has been shown to be significantly homologous with the sequences of the protease inhibitors ovalbumin and antithrombin III (31, 32). Complementary DNA studies on the human and baboon α_1 -AT further substantiate these similarities (33). A fascinating report has recently been published of a heterozygote with Pi M Philadelphia (34). In the Philadelphia mutant the methionine at position 358 was substituted by an arginine, thus mimicking the active site of antithrombin III, with serious bleeding during acute phase reactions as a result.

The Pi Z Deficiency State

Clinical interest was first focused on α_1 -AT with the discovery of premature panlobular basal emphysema in the Pi Z state (1). Later research has established the proteolytic action of elastase and cathepsin G from leucocytes and alveolar macrophages on elastin in alveolar tissue (35) and the protective roll of α_1 -AT (36). More recently the protease-antiprotease imbalance theory has been expanded by the discovery of decreased protease inhibitor capacity of α_1 -AT subsequent to the oxidation of methionine at position 358 (37). Such oxidation has been demonstrated in the presence of cigarette smoke and in the presence of enzymes released by leukocytes and macrophages. The additive effect of α_1 -AT deficiency and oxidation has also been biologically demonstrated by the decrease in elastic recoil found in smoking MZ heterozygotes as compared to non-smokers (38). Thus the protease-antiprotease imbalance concept which was based on the discovery of emphysema in Pi ZZ individuals has contributed to a better understanding of emphysema occurring in smoking Pi M subjects with frequent upper respiratory infections.

The association of liver disease and the deficiency state was first noted by Sharp et al. in 1969 (2) with the discovery of juvenile cirrhosis in one of two siblings with the deficiency. Immunoreactive globular inclusions of α_1 -AT were later identified by electron microscopy in the rough endoplasmic reticulum (RER) of hepatocytes of several such patients (3) and found to correlate with PAS-positive globules seen on light microscopy. Chemical characterization of such globules has shown essentially pure α_1 -AT (39) with the same amino-terminal sequence as the normal plasma protein (40), an unexpectedly high mannose content and absence of sialic acid (41).

In a prospective screening program, Sveger has found 120 infants with phenotype Pi ZZ among 200,000 Swedish infants (42). Of

these infants 14 had neonatal cholestasis, 8 had signs of liver disease without jaundice, and nearly 50% had elevated alanine-amino transferase or γ -glutamyl transferase levels. After 8 years, 2 children with neonatal cholestasis have died of cirrhosis and 2 others with other causes of death have had liver cirrhosis or fibrosis evident at autopsy. One Pi SZ child has died of cirrhosis. About 40% of the Pi ZZ children have abnormal liver function tests at 8 years of age (43).

Many studies correlating α_1 -AT deficiency with adult liver disease have been performed with results indicating a high prevalence of cirrhosis and hepatoma (4, 5) to results suggesting no or slight correlation (44, 45, 46). Several anecdotal case reports of cirrhosis or hepatoma in the Pi MZ, SZ or MS phenotypes have been published and are reviewed by Sharp (47). One retrospective study on 1,055 liver biopsies using the presence of PAS-positive globular inclusions as a marker for the Pi Z allele, has suggested that the heterozygous Pi MZ state also predisposes for chronic active liver disease and cryptogenic cirrhosis (6). Thus far no consensus has been reached as to the probable pathogenesis of liver disease in the deficiency state. The absence of liver disease in the rare Pi -- and the Pi S phenotypes, however, suggests that the intracellular aggregates may be of central importance. By investigating intra- and extracellular levels of α_1 -AT in a population with liver disease as well as the distribution of phenotypes in this group we have hoped to gain insight into this problem.

ALPHA₁-ANTITRYPSIN IN LIVER DISEASE

Plasma Levels of α_1 -AT in Liver Disease (I)

Increased levels of α_1 -AT had previously been reported in liver cirrhosis (48) and a pattern of acute phase reactant proteins with high α_1 -AT, normal orosomucoid and low haptoglobin had been described for acute viral hepatitis when this study was initiated (49). Our goal was to correlate specific features seen in liver biopsy specimens with plasma α_1 -AT levels and to compare concentrations of α_1 -AT to other plasma proteins under such conditions.

Histology, clinical data and simultaneous plasma protein analyses were evaluated from 202 consecutive patients undergoing liver biopsy at Malmö General Hospital according to routine clinical indications. Sixteen "healthy controls" were also biopsied after informed consent during elective cholecystectomy.

Only patients judged to have primary liver disease were included in statistical calculations. Each liver biopsy was evaluated microscopically and given a score from 0-3 for each of 10 biopsy characteristics and for the subjective impression of inflammatory activity to give a total score of 0-33 points. α_1 -AT levels increased significantly with increasing inflammatory activity and total biopsy score (Table I:I). Specific biopsy characteristics associated with this increase were piece-meal necrosis, spotty lobular necrosis, Kupffer cell activity, bile duct replication and round cell portal infiltration in decreasing order of significance. No correlation was found with fibrosis, cirrhosis, steatosis, cholestasis or bridging necrosis. In the last case this was probably due to the small number of biopsies displaying this feature (Table II:1). Mean α_1 -AT levels were higher in each diagnostic group than in controls with the highest levels in patients with viral hepatitis, chronic active hepatitis and

alcoholic liver disease (Table III:I). In the total material a tendency toward an acute phase reactant pattern was seen with an increase in α_1 -AT, no change in orosomucoid and a decrease in haptoglobin with increasing total score and activity (Table I:I). This pattern was found to have a low sensitivity (0.29), a high specificity (0.96) and a moderate predictive value (0.71) for liver disease when compared with 250 consecutive plasma protein analyses regardless of diagnosis. The presence of the acute phase reactant pattern in the absence of liver disease was nearly always due to estrogen administration.

Intracellular α_1 -AT (II)

Liver biopsies from 228 patients and 22 controls were stained by PAS after treatment with diastase and by an immunoperoxidase technique (50) specific for α_1 -AT. In each case intrahepatocellular α_1 -AT was recorded as absent, present as diffuse or granular deposits in the cytoplasm, or as globular inclusions. Appearance of α_1 -AT in sinusoidal liver cells was also noted when present. Phenotypes were determined by IEF. Hepatocytes in only 28 of 227 subjects lacking the Z allele had diffuse or granular α_1 -AT in their hepatocytes. The mean plasma α_1 -AT level in these patients was slightly higher than in patients with negative biopsies ($p < 0.33$). Their mean biopsy score was 7.6 ± 5.1 compared to 5.3 ± 4.4 ($p < 0.05$). Inflammatory activities were 1.4 ± 1.0 and 1.0 ± 0.9 for the two groups respectively ($p < 0.05$). Biopsies from 102 patients showed positive α_1 -AT staining in leukocytes, Kupffer cells or round cells but no differences were found in mean plasma α_1 -AT concentration, biopsy scores or inflammatory activities between these patients and those with negative biopsies.

Globular α_1 -AT inclusions were found in 11 of the 15 cases (including controls) who carried the Pi Z allele for α_1 -AT deficiency. One Pi MZ case stained diffusely positive and 3 were negative. Negative cases may be explained by small biopsy size

and uneven distribution of the inclusions. Globular inclusions were also found in 3 cases of Pi M of which only one could be regarded as typical congestive globules (51). When comparing with the other cases of α_1 -AT globules in the absence of Pi Z in the literature (44, 52, 53), high plasma levels and active, frequently terminal, disease were found to be common features. Whereas amino acid substitutions resulting in no net change of iso-electric point as in Pi M Malton (54) but increased aggregating tendency as in Pi M Duarte (55) cannot be excluded in these cases, the high plasma α_1 -AT levels suggest that a secretory defect is unlikely. We have hypothesized that during lively synthesis, the rate of translation of α_1 -AT may exceed a rate-limiting step of glycosylation resulting in intracellular accumulation.

Distribution of Phenotypes among Patients with Liver Disease (II, III)

IEF was performed on sera from all patients undergoing liver biopsy from August 1978 to April 1980. The distribution of phenotypes among 228 patients is shown in Table I:II. A slight but insignificant ($p>0.1$) overrepresentation of the Pi Z gene was found compared to normal Scandinavian populations, but no differences among these patients could be found regarding age, total biopsy score, inflammatory activity, fibrosis or cirrhosis compared to other phenotypic groups.

We were therefore rather surprised at the results of a large study from Southampton in which Hodges et al (6) found the phenotype Pi MZ in about 20% of all patients with non-B chronic active hepatitis and cryptogenic cirrhosis. For this reason the study was expanded to include all patients undergoing either liver biopsy or the aminopyrine breath test for evaluation of microsomal liver cell function (56) during a five-year period (III). Plasma samples from 861 patients were screened using a monoclonal

antibody against Pi Z α_1 -AT in an enzyme-linked immunosorbent assay (ELISA) method. This antibody had been prepared after immunization of mice with globular α_1 -AT inclusions purified from a Pi ZZ liver (40) according to standard methods (57). A clone was found to produce antibodies which failed to recognize Pi M, S or F α_1 -AT but consistently recognized Pi Z (58). All samples identified by this antibody and an equal number of non-Pi Z controls were subjected to IEF for phenotype determination.

Four patients had phenotype Pi ZZ and 64 (7.6%) were heterozygotes for Pi Z compared to an expected 4.8% ($p < 0.001$). The sex ratio of heterozygotes with liver disease was 2 males: 1 female as has previously been seen among asymptomatic Pi ZZ children with elevated serum enzymes (42) and in Pi ZZ adults with cirrhosis and hepatoma (5). This ratio may be compared to an equal sex distribution within population studies (59) and 58% males among the total study population ($p < 0.001$).

To evaluate the nature of liver disease among heterozygotes, data concerning clinical, laboratory and biopsy features were collected from medical records and compared to corresponding data from twice as many age- and sex matched controls from the same study population. Special interest was focused on the probable etiology of liver disease and its severity. Alcohol was more common as a causative factor in controls than heterozygotes ($p < 0.01$). Liver disease was regarded as cryptogenic in at least 14 heterozygotes compared to 3 controls ($p < 0.001$). The severity of liver disease was generally slightly greater in heterozygotes. Malignant hepatoma was found in 6 of 64 heterozygotes compared to 1 of 128 controls ($p < 0.01$) and 13 of all 793 non-Pi Z patients in the study population ($p < 0.001$).

We conclude that intermediate α_1 -AT deficiency predisposes for chronic liver disease, constitutes a considerable subgroup of apparently cryptogenic liver disease, and increases the risk for primary liver cancer in patients with established liver disease.

The results of this study together with that of Hodges et al (6) suggest that the intracellular inclusions of Pi Z α_1 -AT may be central in the pathogenesis of liver disease associated with the Pi Z allele as 51% of these patients had plasma α_1 -AT levels within the normal range. To gain further insight into the possible mechanisms of liver disease, we have investigated the intracellular steps of the biosynthesis and secretion of α_1 -AT.

INTRACELLULAR PROCESSING OF PROTEIN PRECURSORS

The Signal Hypothesis

In 1971 Sabatini and Blobel (60) and in 1972 Cesar Milstein (61) independently proposed a signal at the NH_2 -terminal of proteins promoting association between ribosomes and endoplasmic reticulum membranes. It is now known that cytoplasmic proteins are formed by the translation of mRNA on free ribosomes and have hydrophilic NH_2 -terminal amino acid sequences, but that mRNA for secretory proteins contains the code for an NH_2 -terminal sequence of mainly hydrophobic amino acids in a similar but variable pattern (62). When this sequence has been translated and extends beyond the ribosomal surface it is recognized by a receptor complex on the endoplasmic reticulum surface (63, 64) which ensures penetration of the membrane and sequestration of the nascent protein into the channels for protein export (65). When access to the endoplasmic reticulum lumen has been attained, the signal is removed by signal peptidase (66) and translation proceeds to the carboxy-terminal of the secretory protein in the functional unit of ribosome plus endoplasmic reticulum (RER) thus established (Fig 2).

The Signal Hypothesis

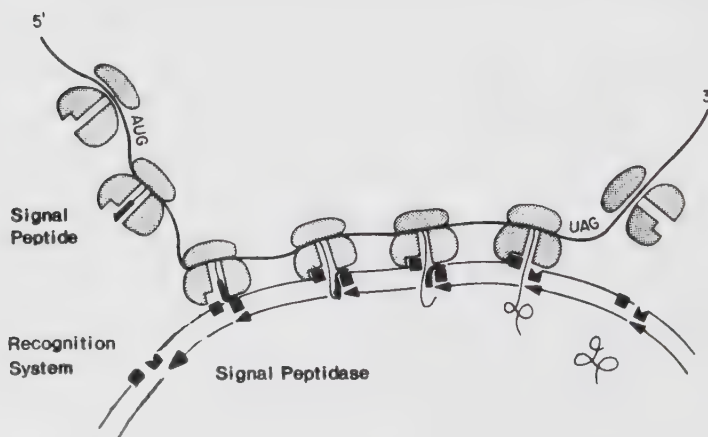


Figure 2. The mRNA translation of a secretory protein . For explanation see text.

By 1980 it was known that insulin had a precursor containing a centrally located proinsulin (67) and that several pituitary peptide hormones were translated in series interrupted by short sequences of mainly basic amino acids proteolytically removed in conjunction with hormone secretion (68). Albumin, the only mammalian liver protein whose total signal sequence had been reported had been shown to be synthesized with a signal peptide of 18 mainly hydrophobic amino acids, followed by a hexapeptide or "pro-peptide" of 6 mainly basic amino acids (69). Its signal peptide is removed contrtranslationally in the RER but the propeptide remains attached and is removed by Cathepsin B in conjunction with exocytosis from the secretory granules (70).

Glycosylation

Aside from albumin, nearly all plasma proteins are glycoproteins the majority of which are formed by N-glycosylation (71). The first step of this process termed "core-glycosylation" occurs during translation in the RER (72) and is summarized in Figure 3.

CORE GLYCOSYLATION

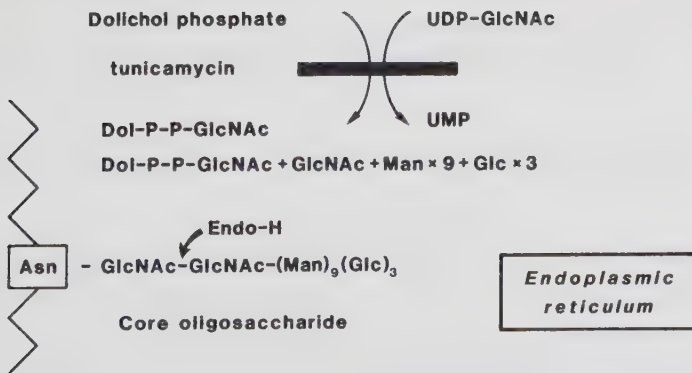


Figure 3. The first step of N-glycosylation, see text.

Core glycosylation can be blocked by the antibiotic tunicamycin as shown (73). The enzyme endo-N-β-acetylglucosaminidase H (endo-H) selectively removes core-oligosaccharides (74).

The high-mannose core oligosaccharide is subsequently trimmed, one sugar residue at a time by selective enzymes, mainly in the smooth endoplasmic reticulum (SER), but with the final mannosidases present in the cis-Golgi. There the glycoproteins interact with a series of specific glycosyl transferases resulting in the final complex glycoprotein (75). Sialic acid, the only electrically charged residue, is added in the trans-Golgi as the final step (76) (Fig 4).

TERMINAL GLYCOSYLATION

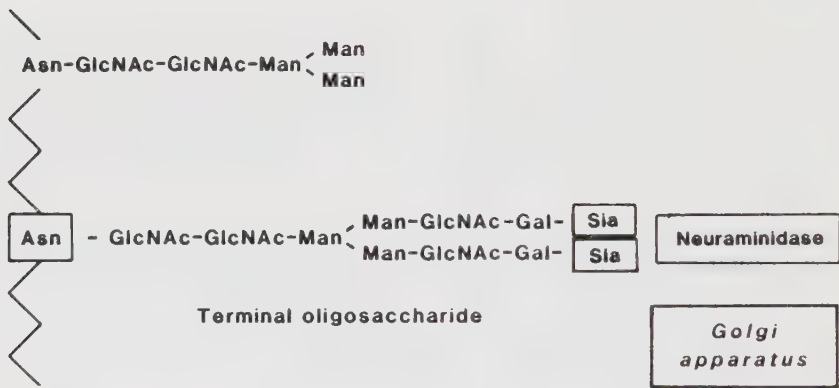


Figure 4. Modification of oligo-saccharide chains, see text.

In the process of trimming and complex glycosylation, variations in the branching of the oligosaccharide occur. Probably the most common chain form is a biantennary oligosaccharide, but triantennary, tetraantennary and hybrid forms (75) are known to exist (Fig 5). A summary of the steps of glycoprotein synthesis is shown in Figure 6.

Branching of Oligosaccharide Chains

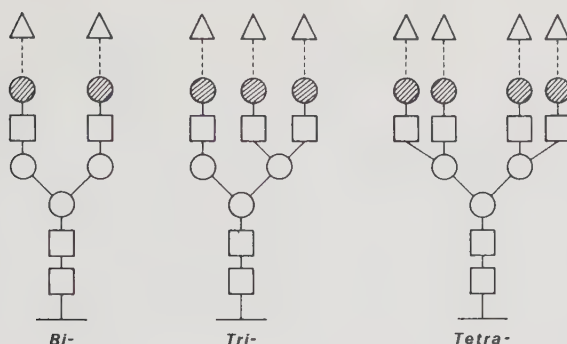


Figure 5. Structures of bi-, tri- and tetraantennary oligosaccharides.

□ = GlcNAc, ○ = Mannose, ◐ = Galactose, △ = sialic acid.

Oligosaccharide structures may serve as signals for subcellular compartmentalization in the cell. These structures along with the primary amino acid sequence dictate the succession of post-translational modifications which give proteins their special functions such as phosphorylation of lysosomal enzymes (77), carboxylation of Glu-residues in vitamin K dependent coagulation factors (78) and the formation of disulfide bonds (79).

Protein Synthesis

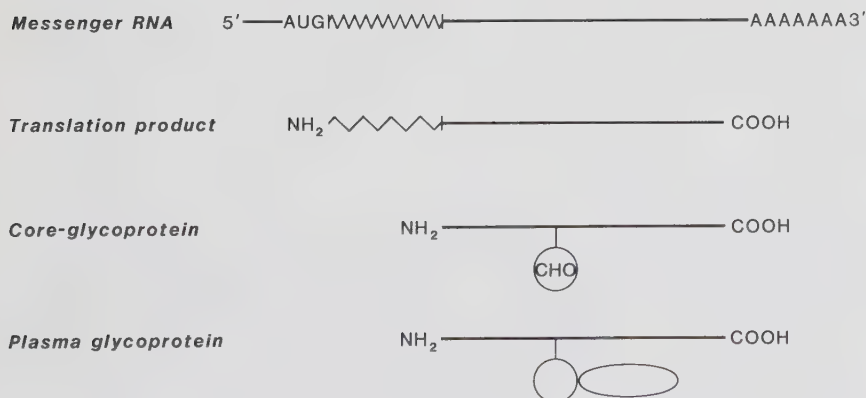


Figure 6. A summary of the synthetic steps of export glycoproteins, see text.

THE BIOSYNTHESIS OF ALPHA₁-ANTITRYPSIN

Aside from the puzzling intracellular accumulation of Pi ZZ α_1 -AT, this study was prompted by the observation of multiple cathodal peaks on crossed immunoelectrophoresis of human fetal liver tissue explant homogenates (80). We knew of the pre- and pro- peptides present in albumin and considered the extra cathodal peaks to be possible pre- or pro- forms of α_1 -AT, intracellular complexes, or possibly degradation products. This study was initiated to characterize the intracellular precursors of α_1 -AT and to follow the events occurring during the process of secretion.

The In Vitro Translation of Rat Liver mRNA (III)

Rat liver mRNA was prepared by the method of Chirgwin et al. (81) with affinity chromatography on oligo-dT-cellulose support which takes advantage of the unique content of poly-adenyl residues at mRNA's 3' terminal end.

In vitro mRNA translation was performed in a reticulocyte lysate system. Reticulocytes contain ribosomes and other nutrients necessary for protein translation, but lack the channels for protein export. Red cells from rabbits with hemolytic anemia were washed and lysed. Native mRNA, coding mainly for hemoglobin, was destroyed by incubation with the calcium dependent staphylococcal nuclease and enzyme activity was interrupted by the addition of EGTA (82).

The lysate solution was then enriched with transfer RNA (tRNA), cofactors and a solution of 19 "cold" and 1 radioactively labeled amino acid. Total rat liver mRNA was added and translation proceeded for 90 min at 37°C. After this incubation the solution contained all the initial ingredients as well as a mixture of radioactively labeled polypeptides normally produced by

rat liver. Rat α_1 -AT was then immunoprecipitated from the mixture onto Protein A Sepharose, eluted and visualized (along with total translation products) by autoradiography with fluorographic enhancement of polyacrylamide slab electrophoresis gels.

By repeating this procedure with each one of the twenty amino acids as the single radioactively labeled amino acid, material was obtained for sequencing studies. The results of this study showed the presence of a signal peptide with 24 amino acids but no sequence with basic amino acids corresponding to the propeptide of albumin.

Rat mRNA Translation in the Presence of Canine Pancreas Microsomal Vesicles (IV)

Endoplasmic reticulum membranes from canine pancreas can be prepared in the form of microsomal vesicles (83). If mRNA is added to a translation mixture containing such vesicles, export proteins should be translocated across the membrane where signal peptides are removed and core-glycosylation occurs. Figure 2:IV demonstrates that the α_1 -AT mRNA translation product in the absence of vesicles has an M_r of 43,200 Daltons and is susceptible to degradation by proteinase K in the absence of detergent. When mRNA is translated in the presence of vesicles, the α_1 -AT produced has an M_r of 52,000 Daltons and is resistant to proteinase digestion indicating transport of the molecule across the endoplasmic reticulum membrane into the vesicle lumen. After disruption of the vesicle membrane by detergent the protein is susceptible to degradation by proteinase K. From the differences in M_r of digestion products it can be deduced that an NH_2 -terminal peptide with M_r of approximately 3,000 Daltons has been removed from the vesicle product and that addition of another molecular moiety with M_r of 9,700 Daltons has occurred.

In Vivo Synthesis and Secretion of Rat α_1 -AT (IV)

To further delineate the post-translational modifications of rat α_1 -AT, pulse chase experiments in isolated rat hepatocyte culture were performed. In such experiments cells are cultured in a serum free medium containing 19 unlabeled amino acids and other factors necessary for cell growth. One radioactively labeled amino acid, in this case ^{35}S -methionine is then added and protein synthesis can be followed by studying the radioactive immunoprecipitable protein in cell lysates or medium at various time intervals after addition of the isotope.

In this system radioactive α_1 -AT with M_r of 50,000 Daltons could be immunoprecipitated from cell lysates 15 min after addition of ^{35}S -methionine to the cell culture medium. Fifteen min later a form with M_r 52,000 Daltons could be identified intracellularly. This latter form was apparently identical to the α_1 -AT secreted from the cells and precipitable from the medium 45 min after addition of the isotope to culture. Further studies of these two intracellular forms showed that the 50,000 Daltons species was susceptible to digestion with endo-H and partial digestion products demonstrated 3 oligosaccharide side chains in the "high-mannose" or core-glycosylated state. This protein was not altered by treatment with neuraminidase and was not secreted from the cells. The second form with M_r 52,000 Daltons was resistant to endo-H digestion, susceptible to neuraminidase treatment and identical in these respects to the α_1 -AT collected from cell culture medium.

In the presence of tunicamycin α_1 -AT with M_r 41,000 Daltons was formed. This corresponds to the translation product in the reticulocyte lysate system minus its signal peptide and indicates an M_r of about 9,000 Daltons for the carbohydrate moiety of the core-glycosylated form.

From these experiments we could conclude that rat α_1 -AT is

synthesized independently of other proteins with a signal peptide containing 24 mainly hydrophobic amino acids, that it has two stable intracellular forms; first the core-glycosylated and later a terminally glycosylated form and that α_1 -AT does not have an NH_2 -terminal propeptide analogous to that of albumin. Furthermore, the secretion of α_1 -AT to the culture medium demonstrates that the total synthesis and secretion of α_1 -AT requires about 45 min and that glycosylation is not an absolute prerequisite for export.

The biosynthesis of α_1 -AT by a human hepatoma cell line (V)

The PLC/PRF/5 human hepatoma cell line has been shown to accumulate intracellular aggregates of α_1 -AT of similar appearance to the globules found in Pi Z hepatocytes (7). Our aim in studying this cell line was twofold being 1) to delineate the biosynthetic steps of human α_1 -AT and 2) to gain insight into the secretory defect in the Pi Z state.

After short periods of subculture no α_1 -AT globules could be seen in the PLC/PRF/5 cells but after 10 days of subculture about 50% of the cells had globules that could be demonstrated in PAS or immunoperoxidase staining. Dilated endoplasmic reticulum was found on electron microscopy of these cells.

We found that the PLC/PRF/5 cell line does secrete biologically active α_1 -AT and that this synthesis and secretion was insensitive to regulation by the addition of exogenous α_1 -AT, colchicine, estrogen or estrogen plus human sex hormone binding globulin.

The major steps and the time scale for post-translational modifications of human α_1 -AT were parallel to those found for the rat. A radioactively labeled core-glycosylated species with M_r 52,000 Daltons was present in the cells 10 min after the addition of ^{35}S -methionine and was subsequently converted to the complex

or terminally glycosylated form with M_r of 56,000 Daltons.

Although α_1 -AT exported from the PLC/PRF/5 cells had the same M_r and elution volume on gel chromatography as a normal plasma pool, it had a lower sialic acid content than Pi M α_1 -AT. Further analysis by Concanavalin A affinity crossed immunoelectrophoresis explained the altered α_1 -AT appearance by demonstrating a higher degree of branching in the carbohydrate side chains than is normally found.

Intracellular aggregates of PLC/PRF/5 α_1 -AT resembled PiZ globular inclusions in appearance and localization, but the hepatoma cell product was not recognized by a monoclonal antibody specific for the Pi Z mutation site. This observation, together with the abnormal glycosylation of PLC/PRF/5 α_1 -AT, suggests different mechanisms of intracellular aggregation in the two cases. Nevertheless this cell line may be useful for further studies on biosynthetic mechanisms and regulation.

GENERAL DISCUSSION

α_1 -AT has been shown to be translated with a signal peptide of 24 mainly hydrophobic amino acids which is removed after translocation across the endoplasmic reticulum membrane where core-glycosylation occurs (IV). Three oligosaccharide side chains are added and are modified by rapid sequential steps to the terminally glycosylated forms prior to secretion (IV, V). These results have been confirmed by other authors (84-86). There is no evidence to suggest that α_1 -AT is synthesized in series with other proteins in analogy to the peptide hormones (68). Neither is there evidence for the presence of a propeptide sequence at the NH_2 -terminal as in the case of albumin (69) or within the molecule as for insulin (67). α_1 -AT appears to be successively secreted from cells with no storage stage in secretory granules prior to export.

Plasma concentrations of α_1 -AT increase selectively and reflect the degree of inflammatory activity in liver disease (I). This is presumably due to an increased rate of synthesis. In a previous study α_1 -AT biosynthesis in human fetal liver tissue explants was shown to increase after addition of purified human plasma α_1 -AT to the culture medium (80). Similar findings have been reported for the synthesis of prothrombin in hepatoma cell culture, particularly after addition of an NH_2 -terminal proteolytic fragment of prothrombin to culture medium (87). The addition of purified human α_1 -AT to PLC/PRF/5 hepatoma cell culture did not alter the synthesis rate (V), possibly due to the lack of proteolytic enzymes from leukocytes or macrophages or to loss of regulatory mechanisms in the malignantly transformed cells.

It nonetheless seems reasonable to hypothesize feed-back induction as a regulatory mechanism in the synthesis of α_1 -AT mediated by a proteolytic product (possibly the carboxy terminal of α_1 -AT) which signals periferal consumption of the active protein.

If such is the case, local proteolysis due to inflammation in the liver with release of proteolytic fragments adjacent to hepatocyte membranes would result in a more dramatic stimulus of α_1 -AT synthesis than inflammation in distant organs with dilution and/or glomerular filtration of proteolytic fragments on their pathway to the liver.

The concept of feed-back induction is also compatible with the common clinical observations that Pi ZZ livers are not filled to the bursting point with aggregated α_1 -AT inclusions and that plasma α_1 -AT levels increase only slightly in Pi ZZ individuals during acute phase reactions. In contrast, Pi MZ heterozygotes succeed in raising their plasma α_1 -AT levels from 57% of the normal to nearly twice normal levels in extreme acute phase situations (III).

Some patients with liver disease had evidence of α_1 -AT present in the Kupffer cells on immunoperoxidase staining of liver biopsy specimens (II). Although synthesis of α_1 -AT has been reported in other cells than hepatocytes (88), I am not aware of any unequivocal experiment showing de novo α_1 -AT synthesis in Kupffer cells. On the other hand, Kupffer cells are thought to remove α_1 -AT-protease complexes from the circulation (30) and they have been shown to contain asialoglycoprotein receptors in their cell membranes (89). The role of Kupffer cells in processing protease-inhibitor complexes and releasing a putative mediator of the biosynthesis of α_1 -AT merits further investigation.

Diffuse or granular α_1 -AT deposits were also seen in hepatocytes of many patients with liver disease (II). We had expected the immunoperoxidase staining to reflect the current degree of biosynthesis but findings were inconstant and correlated only weakly with plasma α_1 -AT levels. This may be due to protein loss during fixation and staining of the thin tissue sections. In exceptional patients with high levels of Pi M α_1 -AT, globular aggregates indistinguishable from Pi Z globular inclusions were

found in hepatocytes. We suggested that this might be due to a rate limiting step in the glycosylation process (II). Fitting and Kabat (90) have suggested a glycoprotein transport molecule in the RER facilitating transport of some glycoproteins to the sites of terminal glycosylation. If their hypothesis is correct, Pi M aggregates in the RER could be explained by saturation of binding sites on the transport vehicle.

α_1 -AT globular inclusions unrelated to the Pi Z gene were also found in the PLC/PRF/5 human hepatoma cells. These cells secreted abnormally glycosylated α_1 -AT with decreased sialic acid content and increased carbohydrate branching compared to normal Pi M α_1 -AT (V). Decreased sialylation of glycoproteins has been reported in hepatomas (91), other malignancies (92) and advanced liver disease (93), possibly due to altered glycosyl transferase activity. Such glycoprotein modifications may hamper interpretation of the complex IEF pattern when determining α_1 -AT phenotypes, i.e. Pi M α_1 -AT with variable sialylation may resemble Pi MZ. A monoclonal antibody specific for the Pi Z mutation site (58) or new methods utilizing complementary DNA technology (94) should resolve controversies as to the actual presence of the Pi Z gene in such cases or in cases of non Pi Z globular inclusions.

Such a monoclonal antibody proved useful in the rapid screening of plasma samples from 861 patients with liver disease for presence of the Pi Z gene (III). The results of this study showed a significant overrepresentation of intermediate α_1 -AT deficiency among patients with liver disease. Although Pi Z globular inclusions were not sufficiently conspicuous to be routinely noted by pathologists in these patients, they could be found when specifically sought. The association of liver disease with both homozygous and heterozygous Pi Z α_1 -AT deficiency and the absence of liver disease in the rare Pi -- and the Pi S phenotypes suggests that the Pi Z molecular structure and/or globular inclusions are central in the pathogenesis of Pi Z related liver disease.

The studies in this thesis have not succeeded in explaining mechanisms behind the secretory defect of Pi Z α_1 -AT, but it is now clear that the globular inclusions consist of core-glycosylated α_1 -AT that has not proceeded to the sites of terminal glycosylation and export. The selective increase in α_1 -AT synthesis secondary to inflammation in the liver (I) causes an increasing accumulation of Pi Z α_1 -AT in the periportal cells which are regarded as having a specialized defensive function in the liver lobulus. This has been termed the recruitment-secretory block phenomenon by Callea (95). One may speculate that engorgement of the RER with aggregated α_1 -AT inhibits the translation and flow of new glycoproteins destined for the plasma membrane, resulting in ageing and disruption of the cell membrane with subsequent necrosis. Phagocytosis of the necrotic cells and associated phenomena may lead to further release of proteolytic products. Thus the hypothetical feed-back induction is perpetuated with recruitment of additional hepatocytes to lively α_1 -AT synthesis and potential cell death. In this manner mild or temporary exposure to exogenous toxins may initiate a chronic low-grade but progressive process in the livers of Pi Z patients, causing chronic "cryptogenic" liver disease.

The Pi Z gene is present in 4.8% of Scandinavians who appear to have an increased risk for liver disease. Many of the Pi Z heterozygotes in our study with asymptomatic or mild disease had elevated γ -glutamyl transferase levels. Many had been unjustly accused of excessive alcohol consumption in their contacts with the medical profession, although alcoholism was generally less prominent among heterozygotes than controls. The increased risk for cryptogenic cirrhosis and malignant hepatoma among Pi Z heterozygotes suggests that it may be wise to determine α_1 -AT phenotype in patients with abnormal liver function tests, and when possible, to limit exposure to known hepatotoxic agents at an early stage of Pi Z related liver disease.

GENERAL SUMMARY

This thesis includes clinical studies on the role of α_1 -AT in liver disease and experimental studies of the biosynthesis of α_1 -AT in vitro and in cultured hepatocytes.

Plasma concentrations of α_1 -AT increased significantly with increasing degree of inflammatory activity in concomitant liver biopsy specimens, but were unaffected by the degree of cirrhosis, fibrosis or steatosis (I). These findings are compatible with the concept of feed-back induction in the regulation of α_1 -AT biosynthesis.

The selective increase of plasma α_1 -AT compared to the other acute phase reactant proteins contributes to a typical pattern with low sensitivity (0.29) but high specificity (0.96) for liver disease (I). This pattern, when present, is useful in clinical evaluation of the severity of liver disease.

Diffuse intracellular deposits of α_1 -AT can be seen in some patients with high plasma α_1 -AT levels, but do not accurately reflect biosynthetic rates. Globular, PAS-positive and immunoreactive α_1 -AT deposits were found in 11 of 15 carriers of the Pi Z allele and in 3 of 207 patients with normal Pi M phenotype (II). Such deposits in the Pi M phenotype are thought to represent a rate limiting step in the modification of core-oligosaccharides.

Plasma samples from 861 patients with liver disease were screened for the presence of Pi Z α_1 -AT using a specific monoclonal antibody and IEF was performed on positive samples for determination of Pi phenotype. Four patients had Pi ZZ and 64 (7.6%) were heterozygotes for Pi Z compared to an expected 4.8% ($p < 0.001$). The sex ratio among these heterozygotes was 2 males:1 female. At least 14 of the heterozygotes had cryptogenic liver disease compared to 3 of 128 sex- and age matched controls ($p < 0.001$). Malignant hepatoma was present in 6 of 64 heterozygotes

compared to 1 of 128 controls ($p < 0.01$) and 13 of all 793 non-Pi Z patients in the total study population ($p < 0.001$).

The increased frequency of liver disease in intermediate Pi Z α_1 -AT deficiency and the high rate of hepatoma in heterozygotes with established liver disease suggests that the recruitment of periportal hepatocytes to lively Pi Z α_1 -AT synthesis may result in cell death and lead to the development of chronic progressive liver disease.

Experimental studies showed that rat α_1 -AT is translated with an NH_2 -terminal signal peptide of 24 mainly hydrophobic amino acids. This signal peptide is removed after penetration of the endoplasmic reticulum membrane ensuring access to the channels for protein export. Three core-oligosaccharide side chains are added to the polypeptide during the process of translation and peptide elongation. After trimming and terminal glycosylation of the oligosaccharides, the glycoprotein is exported from the cells. This process is similar in isolated rat hepatocytes and in human hepatoma cells. The total process of biosynthesis and secretion requires about 45 min. Secretion is delayed but not prevented by tunicamycin, an antibiotic which blocks core-glycosylation.

The human PLC/PRF/5 hepatoma cells accumulate intracellular globular deposits of α_1 -AT in the RER in analogy to the human Pi Z deficiency state. α_1 -AT produced in these cells does not react with the Pi Z specific monoclonal antibody and is abnormally glycosylated, suggesting two different mechanisms of α_1 -AT aggregation in these two systems. Nonetheless, this cell line may be valuable in further studies of the regulation of α_1 -ATs biosynthesis and mechanisms of cell damage.

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α_1 -Antitrypsin and other Acute Phase Reactants in Liver Disease

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ABSTRACT. The plasma acute phase reactant pattern was studied in 124 patients with liver disease and 16 healthy individuals undergoing liver biopsy. α_1 -Antitrypsin levels were found to correlate positively with the extent of hepatocellular damage, inflammatory activity and total biopsy score. Haptoglobin levels correlate negatively with these parameters and particularly with characteristics conducive to portal hypertension. Orosomucoid and fibrinogen were unaffected by extent of disease and activity. These changes result in a typical acute phase reactant pattern, seen most frequently in viral hepatitis and chronic active hepatitis and less frequently in alcoholic liver disease. When present, it has a high specificity and predictive value for detection of liver disease.

Key words: α_1 -antitrypsin, acute phase reactants, liver disease, biopsy.

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Increased levels of α_1 -antitrypsin (α_1 -AT) have been reported in individuals of normal Pi phenotype in various diseases of the liver (11, 12). Kindmark and Laurell (5) have demonstrated a particular pattern (high α_1 -AT, normal orosomucoid, low haptoglobin) of acute phase reactant proteins in cases of viral hepatitis B, differing from the typical acute phase reactant pattern found in other inflammatory conditions.

In the present study, plasma levels of α_1 -AT and other acute phase reactants are correlated to the extent of change in biopsy characteristics, using an objective scale for the evaluation of concomitant liver biopsies. Clear diagnostic groups are compared statistically. The pattern of acute phase reactant proteins regarded as typical for liver disease is defined and its predictive significance is evaluated.

STUDY POPULATION AND METHODS

The original material comprises 202 consecutive liver biopsies taken when clinically indicated from patients admitted to Malmö General Hospital. Forty-six patients had alcoholic liver disease (ALD) (16 with Laennec's cirrhosis (LC) and 28 alcoholic hepatitis (AH)). Thirteen patients had primary biliary cirrhosis (PBC), 18 chronic active hepatitis (CAH), 5 chronic persistent hepatitis (CPH), 2 acute viral hepatitis (VH), 2 cryptogenic cirrhosis (CC) in advanced stages, and 4 venous congestion (VC). Thirty-four additional patients had primary liver disease of unclear or complex type. Sixty-two patients, who had either malignancy or systemic disease influencing the plasma protein pattern or whose biopsy specimens were too small for reliable interpretation, were excluded.

Fasting EDTA plasma samples were obtained on the morning after Menghini needle liver biopsy. Control biopsies were obtained, with written patient consent and with the approval of the Ethical Committee of the Medical Faculty, University of Lund, from 16 subjectively healthy patients with normal liver enzymes during elective cholecystectomy. The final study population thus consisted of 140 patients, 16 of whom were healthy controls. In addition, plasma protein patterns of 17 patients with VH (no biopsy) were included for comparison.

All biopsies were evaluated, using only the internal code from the Department of Pathology for identification. Biopsies were judged using Scheuer's Liver Biopsy Interpretation (10) as reference. Each biopsy was given 0–3 points for each of the characteristics: bridging necrosis, cirrhosis, fibrosis, round cell portal infiltration, piecemeal necrosis, spotty lobular necrosis, bile duct replication, cholestasis, Kupffer cell activity and subjective inflammatory activity (2). The latter was roughly judged as follows: 0 = no inflammation, 1 = one-cell necrosis with round cell infiltration, 2 = infiltration of round cells, leukocytes and macrophages, and 3 = diffuse inflammation of all cell types (13). Additional consideration was given extreme anisocytosis, anisokaryosis and degree of hy-

Abbreviations: α_1 -AT = α_1 -antitrypsin, ALD = alcoholic liver disease, LC = Laennec's cirrhosis, AH = alcoholic hepatitis, PBC = primary biliary cirrhosis, CAH = chronic active hepatitis, CPH = chronic persistent hepatitis, VH = viral hepatitis, CC = cryptogenic cirrhosis, VC = venous congestion.

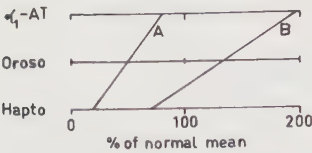


Fig. 1. (A) Pattern of acute phase reactants seen in advanced stages of cirrhosis, independent of origin. (B) Pattern seen in acute viral hepatitis, alcoholic hepatitis and highly active chronic hepatitis.

dropic or eosinophilic changes in hepatocytes, allowing some flexibility in the inflammatory score. Biopsy evaluation was later compared with the pathologist's diagnosis and the score was adjusted after discussion with a pathologist in the few cases where disagreement occurred. Scores were finally complied with clinical and laboratory data from patient charts.

Plasma samples were analysed by agarose gel electrophoresis (4). Albumin was determined by an immunoprecipitate nephelometric technique, α_1 -AT, orosomucoid, haptoglobin, fibrinogen and ceruloplasmin concentrations were determined by electroimmunoassay (6) using a modification in the case of fibrinogen (1). Average values for these proteins determined from a normal plasma pool were 45, 1.35 (3), 0.77, 1.6, 3.2 and 0.35 g/l, respectively. All calculations presented here were performed after converting protein concentrations to per cent of the above average concentrations.

Correlations between biopsy characteristics and plasma protein concentrations were calculated using a program for linear regression. Comparisons of α_1 -AT levels between diagnostic groups and the control group were performed using both the Wilcoxon two-sample test for the unpaired case and the two-tailed Student's *t*-test. The typical pattern of acute phase reactants indicative of liver disease is defined as α_1 -AT concentration minus orosomucoid concentration $\geq 5\%$ and α_1 -AT concentration minus haptoglobin concentration $\geq 20\%$ (Fig. 1). To investigate the sensitivity and specificity of the acute phase reactant pattern for liver disease, 250 consecutive plasma protein analyses in adults from the Clinical Chemistry Laboratory were studied and all patient charts were reviewed.

Table I. Slopes and confidence levels for linear regression curves of acute phase reactant proteins vs. total biopsy score and inflammatory activity

	Total biopsy score	Inflammatory activity
Albumin	-1.01****	-5.74****
α_1 -AT	2.39****	12.34****
Orosomucoid	-0.73 ^{N.S.}	-5.41 ^{N.S.}
Haptoglobin	-3.52***	-18.8***
Fibrinogen	-0.18 ^{N.S.}	-3.03 ^{N.S.}

* 0.05>*p*>0.02, ** 0.02>*p*>0.01, *** 0.01>*p*>0.001, **** *p*>0.001.

Table II. Slopes and significance levels of regression curves for acute phase reactants plotted against biopsy characteristics in 140 cases

No significant correlations were found for bridging necrosis or cholestasis which are therefore omitted. Confidence levels as in Table I

	α_1 -AT	Orosomucoid	Haptoglobin
Fibrosis	5.3 ^{N.S.}	-5.3 ^{N.S.}	-15**
Cirrhosis	4.4 ^{N.S.}	-0.1 ^{N.S.}	-16**
Round cell portal infiltration	7.4*	-8.3*	-15**
Piecemeal necrosis	19***	-5.9 ^{N.S.}	-21*
Spotty lobular necrosis	14****	-2.7 ^{N.S.}	-20**
Bile duct replication	9.9**	-10 ^{N.S.}	-13 ^{N.S.}
Kupffer cell activity	12.4***	-3.6 ^{N.S.}	-3.9 ^{N.S.}
Steatosis	2.6 ^{N.S.}	10.4***	8.6 ^{N.S.}

RESULTS

Total biopsy scores varied from 0 to 23 in the material studied, with a range from 0 to 3 for each of the separate biopsy characteristics. Whereas few biopsies demonstrated bridging necrosis or convincing cholestasis; fibrosis, cirrhosis, portal infiltration, steatosis and inflammation were frequently present. Table I demonstrates the correlation between plasma protein concentration and total biopsy score as well as inflammatory activity for the 140 patients studied. Albumin levels are negatively correlated (*p*≤0.001) with increasing score and activity. α_1 -AT levels covary positively (*p*≤0.001) with these parameters in contrast to orosomucoid and fibrino-

Table III. α_1 -AT levels in different diagnostic groups

Confidence levels as in Table I

	N	α_1 -AT level ^a
Controls	16	101±21
ALD	46	124±35***
LC	16	117±37 ^{N.S.}
AH	28	132±38**
PBC	13	116±16*
CAH	18	139±43***
CPH	5	106±26 ^{N.S.}
VH	19	144±33****
CC	2	145±21 ^{N.S.}
VC	4	147±45 ^{N.S.}

^a % of average normal values ± S.D.

NUMBER OF CASES

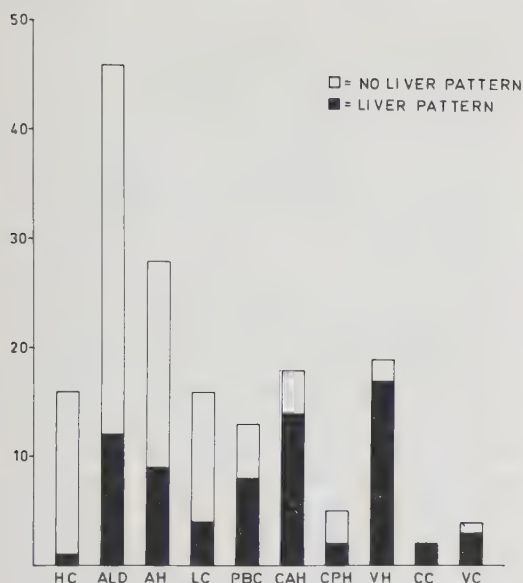


Fig. 2. Presence of the acute phase reactant pattern in different liver disease groups. HC = healthy controls.

gen. Haptoglobin concentrations decrease sharply and significantly ($p \leq 0.01$) but with great variation between individuals with increase in these biopsy characteristics.

Regression lines were similarly calculated for the relationships between α_1 -AT, orosomucoid, and haptoglobin and other biopsy characteristics. The results shown in Table II demonstrate significant increases in α_1 -AT in necrotic and inflammatory conditions and with increasing Kupffer cell activity. Haptoglobin decreases with increasing fibrosis, cirrhosis, portal inflammation and spotty lobular necrosis. Orosomucoid covaries only with increasing level of steatosis.

A comparison of α_1 -AT concentrations in different liver disease groups and controls is given in Table III. Highly significant increases in plasma α_1 -AT were found in cases of AH and VH, significant increases in all forms of ALD and CAH, slight increases in PBC and no significant change in VC or CC. Using the criteria above for the pattern of acute phase reactants typical for liver disease, frequencies of the pattern in the various diagnostic groups are shown in Fig. 2. The frequency of the pat-

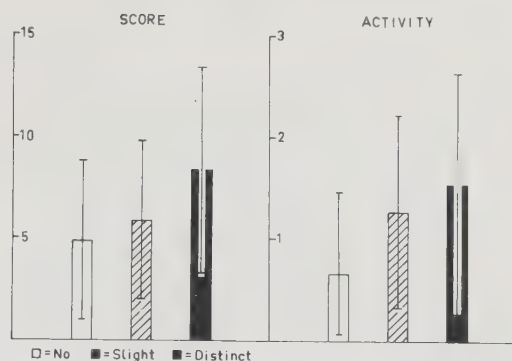


Fig. 3. Increasing liver biopsy score and activity found in patients with no, slight, and distinct patterns of acute phase reactants. Patterns are slight if α_1 -AT minus orosomucoid = 5–14% and α_1 -AT minus haptoglobin $\geq 20\%$, distinct if α_1 -AT minus orosomucoid $\geq 15\%$ and α_1 -AT minus haptoglobin $\geq 30\%$.

tern occurs in the following order: VH>CAH>PBC>AH>alcoholic cirrhosis. In Fig. 3 the acute phase pattern has been subdivided into groups with smaller and greater slopes to demonstrate the tendency toward increased biopsy score and activity in patients with slight and distinct acute phase patterns.

Ceruloplasmin is generally accepted as an indicator of oestrogen effect in the plasma protein pattern (7). To evaluate possible oestrogen effect contributing to the acute phase pattern in liver disease, average ceruloplasmin values were calculated in patients with and without the acute phase pattern. Average values were 0.41 g/l (± 0.13 , $n=48$) and 0.44 g/l (± 0.13 , $n=76$), respectively. No difference in ceruloplasmin levels between those with and without the acute phase pattern could be seen among patients with cholestasis.

The clinical usefulness of the acute phase reactant pattern was investigated using 250 consecutive plasma protein analyses and reviewing the patients' charts. Sensitivity, specificity and the predictive value of the pattern were calculated (Table IV). Of the 250 patients, 69 had a history of current acute or chronic liver disease and/or pathological liver enzymes. Of these 69 patients, 20 satisfied the criteria for the acute phase reactant pattern.

In addition, 8 patients without history of liver disease had the pattern. In this group, 2 patients with metastases of mammary cancer were receiving treatment with the oestrogen receptor blocking

Table IV. Sensitivity, specificity and predictive value of the acute phase reactant pattern typical of liver disease

TP = true positive, FP = false positive, FN = false negative, TN = true negative

	Pats. with a history or elevated enzymes	Pats. with no liver history
Pats. with "liver pattern"	TP 20	FP 8
Pats. lacking "liver pattern"	FN 49	TN 173

Sensitivity = $TP/(TP + FN) = 20/69 = 0.29$.
 Specificity = $TN/(TN + FP) = 173/181 = 0.96$.
 Predictive value = $TP/(TP + FP) = 20/28 = 0.71$.

agent tamoxiphene. Two females with Crohn's disease and one with acute pleuropneumonia, all taking oral contraceptives, also had the pattern. Two female outpatients, 21 and 40 years of age, using no oestrogens and with normal ceruloplasmin levels are included, as is one man with orthopaedic disability but no clinical data concerning liver disease.

DISCUSSION

The results of this study demonstrate a characteristic fall in albumin levels with increasing inflammation and biopsy score. This was expected and validates the histological classification system used. Selective increases in levels of α_1 -AT compared to other acute phase reactants are clearly seen in nearly all diagnostic groups of liver disease. The elevation in α_1 -AT levels corresponds well to total biopsy score and even more markedly to inflammatory activity, indicating its value as an activity parameter. Increased levels in liver disease may reflect decreased catabolism, but more probably indicate an increased synthesis, perhaps in response to local release of proteolytic enzymes from leukocytes and macrophages. It is of interest to note that α_1 -AT levels are related to degree of hepatocellular necrosis, inflammatory cell infiltration and Kupffer cell activity. The elevations of α_1 -AT levels in alcoholic hepatitis are somewhat lower than those reported by other authors (9, 12). This may be because blood samples in our study were taken during convalescence, when liver biopsy was also feasible.

Orosomucoid was nearly unchanged with all biopsy characteristics observed except for steato-

sis. The significant increase in orosomucoid with increasing steatosis is difficult to interpret but may be related to ethanol-induced damage.

The frequent decrease in haptoglobin concentration contributes to the typical pattern of acute phase reactants in liver disease. This decrease may result from portal hypertension with splenomegaly or increased boned marrow hemolysis due to ineffective erythropoiesis among other factors. Interestingly, the haptoglobin level was correlated to degree of fibrosis and/or cirrhosis (Table II), factors conducive to portal hypertension.

The presence of the acute phase pattern typical for liver disease in some patients with oestrogen medication and no clinical or laboratory signs of liver disease invites speculation about the role of hyperoestrogenisation in the plasma protein pattern of liver disease. Clinical signs of hyperoestrogenisation are common in chronic hepatocellular failure (8) and oestrogens are known to increase α_1 -AT levels (7). The normal ceruloplasmin levels in patients with the liver pattern contradict the assumption that hyperoestrogenisation is of importance in evoking this special pattern.

The acute phase pattern for liver disease was found in 29% of unselected patients with a history of current acute or chronic liver disease. The figure of 39% (48/124) among biopsy patients supports the observation of an increased extent of liver disease among patients with the pattern (Fig. 3). The sensitivity of this typical pattern is nonetheless low. This may be partly explained by the large number of patients with alcohol-induced liver disease. As indicated above, the pattern in such disease is blurred by the relatively high orosomucoid level correlating to steatosis. Despite its low sensitivity, presence of the pattern in the absence of exogenous oestrogen is highly specific and predictive for liver disease.

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Intra- and extracellular α_1 -antitrypsin in liver disease with special reference to Pi phenotype

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SUMMARY In order to study the relation between intra- and extrahepatocellular α_1 -antitrypsin (α_1 -AT) concentrations in patients with various Pi phenotypes, a prospective series of needle liver biopsies was stained with both periodic acid-Schiff (PAS) and a specific immunoperoxidase technique to demonstrate intracellular α_1 -AT. Concomitant blood samples from all patients were analysed for α_1 -AT. Pi phenotypes were determined by isoelectric focusing.

Non-globular intrahepatocellular α_1 -AT can be seen in biopsies from Pi M patients with increased plasma α_1 -AT concentrations and active liver disease. No evidence was found in this study of 250 patients (including 22 controls) for predisposition toward liver disease in any phenotypic group. PAS or immunoperoxidase staining (or both) for α_1 -AT demonstrated characteristic globular inclusions in 11 of 15 cases having the Z allele, one case being diffusely positive and three negative. Biopsies from 3 of 207 patients with liver disease and lacking the Z allele had globular inclusions seen with both PAS and immunoperoxidase techniques. α_1 -AT globules in absence of the Z allele are most often found in elderly patients with severe disease and high plasma α_1 -AT concentrations.

Alpha₁-antitrypsin (α_1 -AT, α_1 -protease inhibitor) is an acute phase reactant plasma protein with broad spectrum antiprotease activity synthesised by the hepatocytes. A selective increase of its plasma concentration is often seen in liver disease and this level generally seems to reflect the activity of the disease.¹ Low plasma concentrations are seen in some phenotypes. Of special interest is the Pi Z phenotype in which increased frequencies of fibrosis, cirrhosis and hepatoma^{2,3} have been reported. Neonatal hepatitis occurs in 10% of homozygous Pi Z children and increased hepatic enzyme activity is found in approximately 50% of Pi children.⁴ Typical PAS-positive inclusion bodies, first described by Sharp *et al.*,⁵ and consisting of aggregated α_1 -AT lacking sialic acid and with an amino acid substitution⁶ are seen in Pi Z hepatocytes. Such inclusions were first thought to be pathognomonic for the Z allele⁷ but several reports during the past few years have disputed this assumption. Heterozygous Pi MZ individuals also have globular inclusions and one case of hepatocellular carcinoma in a 16-year-old MZ boy has been reported,⁸ but most investigators have found no evidence of increased liver disease frequency in such individuals.

α_1 -AT may have an important protective role as a protease inhibitor in liver disease. The mechanisms regulating the plasma concentration in both normal and other phenotypes are, however, incompletely known. To gain insight into these mechanisms we have studied (i) the relation between intrahepatocellular immunoreactive α_1 -AT and plasma concentrations in various liver diseases; (ii) the frequency and some clinical conditions associated with globular inclusions (α_1 -AT globules) in absence of the Z allele and (iii) the distribution of phenotypic groups among patients with liver disease. The study was performed prospectively using an α_1 -AT-specific immunoperoxidase staining in addition to routine PAS-staining of 250 liver biopsies (including 22 controls) for visualisation of intracellular α_1 -AT and isoelectric focusing for phenotype determination in all cases.

Patients, material and methods

The material comprises 250 consecutive needle liver biopsies obtained from patients admitted to Malmö General Hospital. It includes 22 control biopsies obtained with informed consent from patients with normal liver function undergoing elective cholecystectomy, according to the principles of the Ethical

Committee of the hospital. The diagnoses were alcoholic liver disease in 88 cases (cirrhosis in 20), chronic active hepatitis (CAH) 15; chronic persistent hepatitis 5; primary biliary cirrhosis 10; venous congestion 6; toxic liver disease 6; hepatoma 6; viral hepatitis 4; systemic disease with liver involvement in 52 cases and diverse or complex diagnoses in the remaining cases. Only one biopsy from each patient was included. Blood samples were obtained from all patients within 24 h of needle liver biopsy for quantitative α_1 -AT and phenotype determinations. Plasma α_1 -AT concentrations were determined at the Department of Clinical Chemistry by an electro-immunoassay technique using a mono-specific antiserum available at the laboratory. Phenotypes were analysed by isoelectric focusing in polyacrylamide gels using a modification of the method of Pierce *et al.*⁹ We thank Dr Jan-Olof Jeppsson for reviewing phenotype designations. All liver biopsies were routinely stained with haematoxylin and eosin, Gordon-Sweet's reticulin, van Gieson's stain and PAS-staining after diastase digestion. Biopsies were judged using only the internal code from the Department of Pathology for identification. Scores were given for the pathological parameters: bridging necrosis, piece-meal necrosis, fibrosis, cirrhosis, portal inflammation, centrilobular necrosis, bile-duct replication, Kupffer-cell activity, steatosis, cholestasis and subjective impression of inflammatory activity.¹⁻¹⁰ Untreated paraffin sections of 242 biopsies were examined separately and independently using the indirect peroxidase conjugate method reported by Taylor.¹¹ Rabbit antiserum, found to be monospecific for human α_1 -AT by crossed immunoelectrophoresis¹² was used in a dilution 1/20 instead of human immunoglobulin components and carbazol was substituted for diaminobenzidine.¹³ Peroxidase-conjugated swine antirabbit IgG was used in a 1/50 dilution. All biopsies which stained positively with this technique were also stained with rabbit antisera against albumin, fibrinogen and IgG. Antisera were obtained from DAKO, Copenhagen, Denmark. Results of the immunoperoxidase examin-

ation were compared with the diastase-treated PAS-stained biopsies.

Results

Distribution of phenotypes and gene frequencies among patients with liver disease compared to Scandinavian normal populations is shown in Table 1. A slight but statistically insignificant ($p > 0.10$) overrepresentation of the Z and S alleles was found among patients with liver disease. No significant differences were found in mean age, total biopsy score, inflammatory activity, degree of fibrosis or cirrhosis between phenotypic groups (Table 2). Two of 11 patients with phenotype MS had chronic active hepatitis (13% of all CAH patients) whereas only 6% of all patients with liver disease had this diagnosis. Subtypes of the M phenotype could easily be distinguished in 14% of M phenotypes after electrofocusing. No evidence was present for any case of M Malton or M Duarte in this material.

Immunoperoxidase staining of α_1 -AT was performed on all 242 biopsies with sufficient material for investigation (including all controls). Hepatocytes in a minority of biopsies (28/227) from patients lacking the Z allele stained diffusely positive for α_1 -AT. Mean plasma α_1 -AT concentrations, biopsy scores and values for inflammatory activity in biopsies were calculated and compared to those for negative biopsies. Among Pi M patients in the positive group the mean α_1 -AT concentration was 1.9 ± 0.7 (SD) and 0.2 g/l higher ($0.32 > p > 0.10$) than for patients with negative biopsies. Mean biopsy score was 7.6 ± 5.1 (SD) compared to 5.3 ± 4.4 ($0.05 > p > 0.01$). Inflammatory activities were 1.4 ± 1.0 and 1.0 ± 0.9 ($0.05 > p > 0.01$) for the two groups, respectively. Biopsies from 102 patients showed positive α_1 -AT staining in leucocytes, Kupffer's cells or round cells, but no differences were found between mean plasma α_1 -AT concentrations, biopsy scores or activities in these and patients with negative biopsies.

Table 1 *Distribution of phenotypes and gene frequencies*

Phenotype	MM	M-	MS	MZ	FM	SS	SZ	ZZ
2830 healthy Norwegians (14)	2540		116	81	72	4	4	2
228 biopsied patients with liver disease	199	1	12	11	3	0	1	1
Gene frequencies	Pi M		Pi S	Pi Z	Pi F	Other		
2830 healthy Norwegians (14)	0.946		0.023	0.0157	0.0133	0.002		
6995 adult Swedes (15)				0.0240				
228 patients with liver disease	0.932		0.0285	0.0307	0.0066	0.002		

The probability that the frequency of the Z gene in liver patients is the same as that among normal Norwegians and Swedes is $0.50 > p > 0.10$ using the χ^2 test. The major contribution to the difference arises in the Z gene group.

Table 2 Correlation between phenotype, liver biopsy characteristics, age and sex among 228 patients with liver disease

Phenotype	n	Mean age	% male	Biopsy score	Biopsy activity	Fibrosis	Cirrhosis
Pi M	199	53 ± 14	59	6.1 ± 4.5	1.1 ± 0.9	0.7 ± 0.9 (107)	0.4 ± 0.8 (48)
Pi M-	1	27	0	0	0	0 (0)	0 (0)
Pi FM	3	52 ± 19	33	7.2 ± 7.4	1.2 ± 0.8	1.0 ± 1.7 (1)	0.7 ± 1.1 (1)
Pi MS	12	51 ± 17	45	6.5 ± 4.7	1.2 ± 1.2	0.8 ± 0.7 (8)	0.1 ± 0.3 (2)
Pi MZ	11	50 ± 15	56	5.9 ± 3.7	1.1 ± 1.0	0.9 ± 1.0 (5)	0.4 ± 1.0 (2)
Pi SZ	1	46	100	0.5	0	0.5 (0)	0 (0)
Pi ZZ	1	39	100	5.5	1.5	0 (0)	0 (0)

Biopsy score is based on the sum of characteristics listed in the text (see ref. 14). Maximal possible score was 30. Maximal degree of the single characteristics activity, fibrosis and cirrhosis is 3 points each. The number of patients within each phenotypic group having biopsy signs of fibrosis or cirrhosis is shown in parentheses.

No statistically significant differences were found between any of the phenotypic groups.

Fifteen cases were found to have α_1 -AT specific globules in the hepatocytes including 10 cases of Pi MZ (two controls) and one Pi ZZ. Of these 11 cases, three MZ biopsies were PAS-negative. Three cases of Pi MZ and 1 Pi SZ were negative to both immunoperoxidase and PAS-staining. Of the 207 cases studied with liver disease and lacking the Z allele, 3 had immunoperoxidase and PAS-positive globules in the hepatocytes. Their clinical data, phenotypes, biopsy scores and plasma α_1 -AT concentrations are summarised in Table 3 together with similar cases from published reports. Globules in our patient with adenocarcinoma had typical appearance for α_1 -AT globules and were present in approximately 10 normal hepatocytes adjacent to metastatic tissue. In the case with venous congestion 50-100 hepatocytes with immunoreactive α_1 -AT of both diffuse and globular appearance were found periportal as well as centrilobularly in one lobulus (Fig. 1). In this case alone staining was also positive for IgG,

fibrinogen and albumin. The third case (rheumatoid arthritis) had globules with appearance and location typical for those seen in Pi Z deficiency (Fig. 2).

Discussion

Opinions on the occurrence of liver disease among MZ individuals are divided. Eriksson *et al.*⁹ reported an increased incidence of cirrhosis in MZ patients in a necropsy study where the presence of PAS-positive material was used as a marker of the Z-gene. Neither immunochemical staining nor determination of plasma α_1 -AT concentrations was performed and therefore, viewing the occurrence of α_1 -AT globules unrelated to the Z allele in severe disease, one cannot exclude a certain overdiagnosis of the MZ state in that study. Morin *et al.*²⁰ have found no increased frequency of the MZ phenotype among patients with liver cirrhosis. Blenkinsopp and Haffenden²¹ find

Table 3 Summary of cases with immunoperoxidase demonstrable α_1 -AT containing globules in hepatocytes in absence of the Z gene

Author (ref)	Phenotype	PAS-staining	Plasma α_1 -AT (g/l)	Age (yr)	Clinical data
Bradfield <i>et al.</i> ^{18*}	M	+	2.3	male 79	Acute pancreatitis patient survived 12 days after biopsy
Fischer <i>et al.</i> ^{17†}	M-	+	1.8	female —	Alcoholic hepatitis no further clinical information reported
Kelly <i>et al.</i> ^{18‡}	3MS S	+— +	0.9	male 56	no further information reported Alcoholic hepatitis patient survived 5 months after biopsy
Present study §	M	+	> 4.0	male 50	Metastatic adenocarcinoma of the pancreas. Patient survived 6 months after biopsy
	M	+	2.3	male 67	Severe cardiac decompensation with venous congestion. Patient still alive
	M	+	2.7	female 76	Highly active rheumatoid arthritis. Patient survived 7 months after biopsy

*Plasma α_1 -AT determined by immunoprecipitation (reference 2.4 g/l) Pi typing by isoelectric focusing.

†M-phenotype deduced in absence of family studies, phenotyping done by starch gel and crossed immunoelectrophoresis. Plasma α_1 -AT by the Mancini technique, reference 2.1 g/l.

‡As for * with reference for P- α_1 -AT 1.8-3 g/l.

§Plasma α_1 -AT determined by electroimmunoassay (reference 0.9-1.70, average value 1.32 g/l), Pi typing by isoelectric focusing.

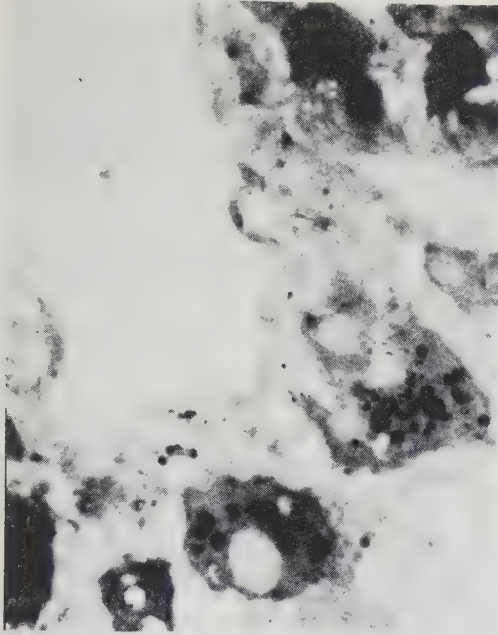


Fig. 1 α_1 -AT containing globules in hepatocytes surrounding the central vein from the Pi M patient with venous congestion (Table 3). Immunoperoxidase staining as described in text $\times 720$.

it probable that Pi MZ is associated with increased fibrosis, cirrhosis and hepatocellular carcinoma (HCC), whereas Kelly *et al.*²² have found no increase in HCC among MZ individuals. In the present

prospective series we find a trend towards overrepresentation of the MZ phenotype among patients with liver disease but it does not reach statistical significance. It should be stressed that absolute comparisons are difficult to perform because of relatively small sample size and diagnostic heterogeneity within phenotypic groups. No increase in mean biopsy score for fibrosis or cirrhosis could be seen in patients with phenotype MZ compared to other phenotypes at similar age (Table 2). Of the 2 MZ patients having cirrhosis, both had a history of alcoholism. No MZ patient lacking history of alcohol overconsumption (3 patients) or cardiac failure (1 patient) had signs of fibrosis. Interestingly an increased frequency of CAH was seen in the Pi MS group. Fisher *et al.*¹⁷ have reported diagnosis CAH among 5 of 18 (28%) Pi MS patients with liver disease. In their study 5/87 (5.75%) of patients with CAH had Pi MS and 18/567 (3.17%) of all patients with liver disease had this phenotype. As they had determined phenotype only in patients with low plasma concentrations of α_1 -AT, and since α_1 -AT is often raised in active liver disease,¹ these figures may be an underestimation. Kueppers *et al.*²³ found the phenotype MS among 8.5% of patients with chronic active liver disease at the Mayo Clinic compared to 3% of healthy blood donors there. These observations together may suggest a predisposition for chronic active liver disease among patients with Pi MS. The plasma concentration is very moderately decreased in these individuals and the mechanism behind such an association is unknown.

The immunoperoxidase staining as well as PAS-

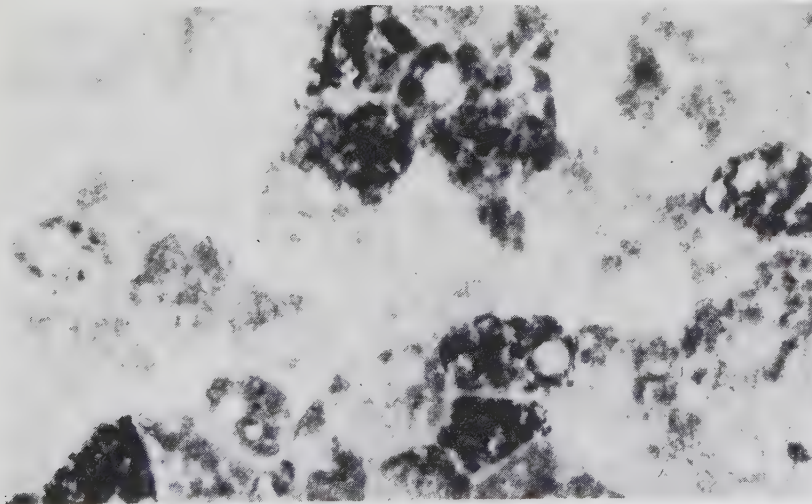


Fig. 2 α_1 -AT globules in hepatocytes of a Pi M patient with rheumatoid arthritis (Table 3). α_1 -AT specific immunoperoxidase staining as described in text $\times 720$.

staining showed diffuse or granular intracellular α_1 -AT deposits in a minority of the biopsies studied. This corresponded to slight increases in plasma α_1 -AT concentrations, total score and inflammatory activity seen in biopsies. Our hypothesis has been that in situations with high plasma α_1 -AT concentrations one should expect high intracellular concentrations reflecting increased synthesis. The regulation of synthesis and secretion, however, may well vary in different diseased states and the intracellular presence of precursor proteins, incompletely glycosylated forms or complexes remains to be studied. Many biopsies probably contain α_1 -AT in concentrations below the threshold of sensitivity for the immunoperoxidase method. In addition, intracellular proteins may be extracted or antigenically altered during treatment of biopsy sections, possibly giving false-negative immunoperoxidase results.

Biopsies from 102 patients stained positively with the immunoperoxidase method for intracellular α_1 -AT in leucocytes, Kupffer's cells (30 cases) or round cells. This may represent degradation products of complex-bound α_1 -AT after phagocytosis but de novo synthesis in these cells, in similarity to that reported in other cells of histiocytic origin,²⁴ cannot be excluded.

The immunoperoxidase method could correctly identify intrahepatocellular α_1 -AT globules in the majority of patients with phenotype MZ and Z, and in that respect was more sensitive than the PAS-staining. Some biopsies from MZ patients had no visible globules with either method. This is not unexpected due to small sample size. More interesting is the occurrence of α_1 -AT globules in 3 cases with the normal M phenotype. Plasma protein analysis, phenotyping and biopsies were evaluated independently in this prospective study of unselected liver biopsied patients. Thus the chance of finding α_1 -AT globules unrelated to the Z gene can be calculated to be 3/207 or 1.4%.

"Congestive globules"²⁵ are well known and are usually easily distinguished from "true" α_1 -AT globules by localisation and appearance. Our case with venous congestion (Table 3 and Fig. 1) probably had such globules, but they were present periportal as well as centrilobularly and could not with certainty be distinguished from α_1 -AT globules. In this case alone globules stained positively even with antisera for fibrinogen, IgG, and albumin.

It appears from our cases and those presented in Table 3 that α_1 -AT globules unrelated to Z gene occur in elderly or terminal patients with high plasma α_1 -AT concentrations reflecting high disease activity. This accumulation may reflect a rate of synthesis exceeding the hepatocyte's capacity for glycosylation or other steps in the secretory process.

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Chronic "Cryptogenic" Liver Disease and Malignant Hepatoma in Intermediate Alpha₁-Antitrypsin Deficiency Identified by a Pi Z Specific Monoclonal Antibody

by

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Using a monoclonal antibody against the Pi Z genetic variant of alpha₁-antitrypsin in an enzyme-linked immunosorbent assay, we have screened plasma samples from 857 consecutive patients with liver disease for the presence of Pi Z alpha₁-antitrypsin. Intermediate alpha₁-antitrypsin deficiency (Pi MZ and SZ) was found in 64 cases or 7.6% compared to an expected 4.8% ($p < 0.001$). The plasma alpha₁-antitrypsin level was subnormal in only 50% of them. Forty-three of the 64 heterozygotes were males (67%) compared to 494 of 857 (58%) in the total study population ($p < 0.001$). No distinctive laboratory or biopsy features were found in heterozygotes compared to 128 sex and age matched non-Pi Z controls from the same study population. Cholestatic features were not prominent. At least 14 heterozygotes had cryptogenic liver disease compared to 3 controls ($p < 0.001$). Malignant hepatoma occurred in 6 heterozygotes compared to 1 control ($p < 0.01$) and 13 of all 793 non-Pi Z patients ($p < 0.001$).

ABBREVIATIONS: AAT = Alpha₁-Antitrypsin; ABT = Aminopyrine breath test; ALAT = Alanine amino transferase; ALP = Alkaline phosphatase; ASAT = aspartateamino transferase; ELISA = Enzyme-linked immunosorbent assay; GT = Gamma-glutamyl transferase; IEF = Iso-electric focusing; PAS = Periodic acid Schiff; PBC = Primary biliary cirrhosis;

INTRODUCTION

Since the association of severe α_1 -antitrypsin (AAT) deficiency phenotype Pi ZZ with familial juvenile cirrhosis by Sharp in 1969 (1), this homozygous deficiency state has been well established as a predisposing factor for adult liver disease, in particular cirrhosis and hepatoma (2, 3). Whereas some studies have denied any association between intermediate AAT deficiency and liver disease (4, 5), one retrospective autopsy study has indirectly suggested an increased prevalence of cirrhosis in heterozygotes for the Z gene by demonstrating periodic acid-Schiff (PAS)-positive, diastase resistant globules in the hepatocytes of such patients (6). Several anecdotal reports of hepatoma in intermediate AAT deficiency have also been published (7, 8). One systematic study has been performed by the meticulous screening of 1 055 liver biopsies from a population of 400 000 for the presence of AAT globular inclusions (9). In this English study, no general overrepresentation of the heterozygous state was found, but about 20% of patients with seronegative chronic aggressive hepatitis or cryptogenic cirrhosis were found to carry the Z allele.

Screening for intermediate AAT deficiency has previously been impractical because of plasma levels which frequently lie well within the normal concentration range coupled with the complexity and expense of phenotyping by starch-gel electrophoresis or isoelectric focusing (IEF). For this reason, a monoclonal antibody which selectively reacts with Pi Z AAT has been developed. It has been used in this study to screen plasma from 861 patients with liver disease for the presence of the Z gene. This study concerns the prevalence of intermediate AAT deficiency among patients with liver disease and attempts to define the spectrum of disease found among these heterozygotes.

MATERIAL AND METHODS

Study population. This prospective study comprises 861 pa-

tients undergoing liver biopsy and/or the aminopyrine breath test (ABT) for the evaluation of liver function at the Department of Medicine, Malmö General Hospital, during a 5 year period. This hospital is the major medical facility in a city of 240 000 inhabitants. Patients with liver disease due to viral hepatitis are primarily treated at the Department of Infectious Disease and are therefore underrepresented as are geriatric patients.

Blood samples. Plasma samples were collected from fasting patients at the time of the ABT or liver biopsy and maintained frozen at -20°C until the time of analysis.

Identification of Pi Z carriers. A monoclonal antibody was prepared after immunization of mice with globular AAT inclusions purified from a Pi ZZ liver (10). A clone was found to produce antibodies which failed to recognize Pi M, S or F AAT but consistently recognized Pi Z (submitted). All samples identified by this antibody in an enzyme-linked immunosorbent assay (ELISA) and an equal number of Pi Z negative samples were subjected to IEF (11) in a single blind procedure. No sample which reacted negatively in the ELISA method could be shown to contain the Pi Z bands on IEF. Four samples which were positive in the ELISA method were falsely negative in the first IEF analyses but on repeated analysis were shown to contain the Pi Z phenotype. Thus the ELISA technique was judged to be more sensitive than IEF and 100% specific for detection of the Z gene.

Control Group and Clinical Data. A list was constructed in chronological order containing birthdates and sex of all 861 patients in the study population and no further data. Two individuals of the same sex and with birthdate nearest that of each heterozygote were selected as controls. Hospital records, including complete autopsy reports for deceased patients were evaluated using a standardized questionnaire to obtain information about history, clinical and laboratory features and outcome.

Laboratory Analyses. The results of the following laboratory analyses which had been performed according to clinical routines

at the time of the first contact with the medical clinic were recorded: bilirubin, aspartate-amino transferase (ASAT), alanine-amino transferase (ALAT), gamma-glutamyltransferase (GT), alkaline phosphatase (ALP), and prothrombin. Plasma proteins had been analyzed by agarose gel electrophoresis (12) and quantitated by electroimmunoassay (13). Values for albumin, AAT, immunoglobulins G, A, and M were recorded. Positive titers for antinuclear antibodies, smooth muscle antibodies, anti-mitochondrial antibodies, and rheumatoid factor were considered when present as were antibodies against hepatitis B surface antigen, cytomegalovirus, and Epstein-Barr virus. Results of the ABT (14) were also registered (reference value 4-8% of ingested ^{14}C -aminopyrine as $^{14}\text{CO}_2$ expired/2 hrs).

Liver Biopsies. Liver biopsies were performed according to clinical indications and routinely stained with hematoxylin-eosin, van Gieson's stain, Gordon and Sweet's reticulin and PAS after diastase digestion. Each biopsy was evaluated by one of several pathologists at the Department of Pathology and results were recorded according to separate parameters as previously reported (15). Biopsies were not routinely screened for PAS-positive globules.

Cause of Liver Disease. Heterozygotes and controls were classified according to the probable etiology of liver disease into several major subgroups. Alcohol related liver disease was assumed in patients with a) a history of heavy alcohol consumption, b) registration at the alcohol clinic or c) a raised GT level judged to be due to heavy drinking (16). Liver disease was considered secondary to systemic disease in patients with connective tissue disease, Crohn's disease, coeliac disease, primary hemochromatosis or metastatic tumor growth when no other cause was apparent. Vascular etiology denotes a history of heart disease and congestion on biopsy. Patients with positive titers for antinuclear antibodies or smooth muscle antibodies and with biopsy findings typical for chronic aggressive hepatitis (17) were con-

sidered to have chronic active hepatitis and were grouped together with patients with a diagnosis of primary biliary cirrhosis (PBC) (18) in the group of autoimmune liver disease. Patients with known acute hepatitis A, acute or chronic hepatitis B or reasonable suspicion of non-A non-B hepatitis are grouped together as virus-related liver disease. A number of patients had mixed etiologies, e.g. moderate alcohol consumption, hepatotoxic drugs, organic solvent exposure, heart failure and/or autoantibodies and are included in a mixed group. Liver disease with no appeared cause was termed cryptogenic.

Severity of Liver Disease. Heterozygotes and controls were further classified into 6 arbitrary categories based on all available data at follow up according to the severity of the disease. They were termed A) asymptomatic when subjectively healthy patients had isolated abnormal laboratory parameters and no hepatomegaly; B) hepatomegaly with enlarged liver on clinical examination or liver scintigraphy without evidence of chronic inflammatory liver disease; C) chronic non-cirrhotic liver disease in cases of chronic aggressive hepatitis regardless of etiology (17) or PBC prior to the development of cirrhosis; D) cirrhosis implies abnormal nodular architecture on biopsy or a clinical constellation compatible with cirrhosis and corresponds to Pugh's groups A and B (19); E) decompensated cirrhosis describes patients with ascites, oesophageal varices and/or portosystemic encephalopathy and corresponds to Pugh's group C and F) malignant hepatoma is reserved for deceased patients with histological evidence of primary hepatocellular or cholangiocellular cancer on autopsy.

Statistical Methods. Laboratory values were compared after calculation of means by t-test. The X^2 -test was used for all other comparisons unless the normal distribution (n.d.) is specified. Non-significant differences are noted as NS.

RESULTS

The study population is described in Table 1. Homozygous AAT deficiency Pi ZZ was found in 4 patients who will not be further discussed. Intermediate AAT deficiency was found in 7.6% of the remaining 857 patients compared to an expected (20) 4.8% ($p < 0.001$, n.d.). Of these 64 patients, 57 (38 men and 19 women) were MZ (expected 4.7%, $p < 0.01$, n.d.) and 7 (5 men and 2 women) were SZ (expected 0.14%, $p < 0.001$, n.d.). Sixty-seven per cent of heterozygotes were male compared to 58% of the total study population ($p < 0.001$, n.d.).

Medical Histories. Family histories were generally incomplete and heredity for liver disease was most common in families with an alcoholic tradition. Parents or siblings of 8 heterozygotes had had fatal liver disease (3 cancer, 5 cirrhosis) of which 3 cases were not alcohol related compared to relatives of 6 controls (2 cancer, 4 cirrhosis) of which 2 cases were unrelated to alcohol (NS).

Table 1. Study Population

		Men	Women
Total number	Total	494 (58%)	363 (42%)
	Heterozygotes	43 (67%)	21 (33%)
	Controls	86 (67%)	42 (33%)
ABT	Total	362 (73%)	294 (81%)
	Heterozygotes	34 (79%)	17 (81%)
	Controls	76 (88%)	40 (95%)
Biopsy	Total	214 (43%)	175 (48%)
	Heterozygotes	26 (60%)	14 (67%)
	Controls	38 (44%)	21 (50%)
Mean age at debut (S.D.)	Heterozygotes	51 (12)	54 (14)
	Controls	52 (12)	54 (13)
Mean observation time yr (S.D.)	Heterozygotes	3.4 (3.5)	3.1 (2.3)
	Controls	2.7 (2.3)	2.9 (3.6)

No patients had knowingly had juvenile cholestasis or cholestasis of pregnancy. One heterozygote had discontinued oral contraceptives because of elevated serum enzyme levels. Three heterozygotes had previously had temporary periods of jaundice of unknown cause.

Connective tissue disease was present in 7 heterozygotes and 8 controls (NS) and chronic obstructive lung disease was found in 8 patients from each group (NS). There was no difference in the presence of renal or other gastrointestinal disease between the

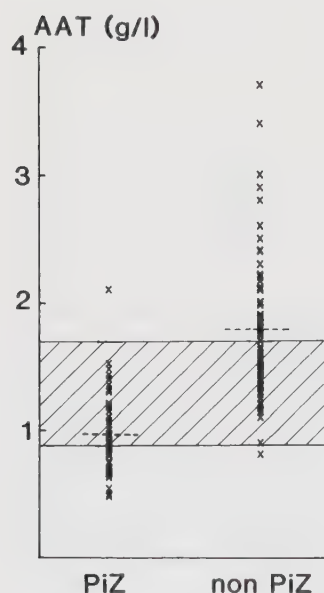


Fig 1. Plasma AAT concentrations in patients with intermediate AAT deficiency and controls. The shaded area indicates the normal range. Dotted lines show means within each group.

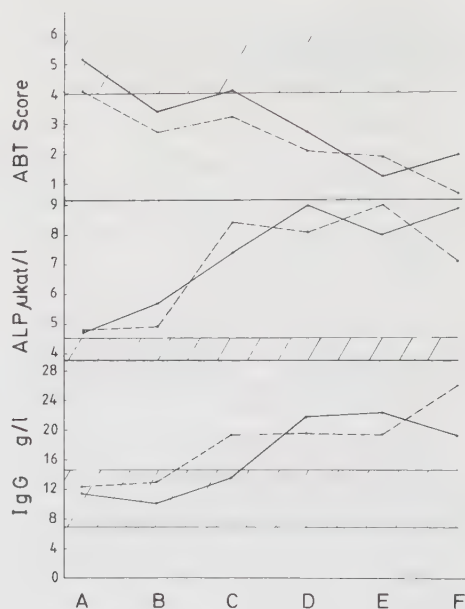


Fig 2. Laboratory parameters according to severity of liver disease in 64 heterozygotes and 120 controls. ABT score = aminopyrine breath test score, normal range 4-8% $^{14}\text{CO}_2/2$ hrs. ALP = alkaline phosphatase, normal range $< 4.6 \mu\text{kat/l}$. IgG = immunoglobulin G, normal range 7-15 g/l.

A = Asymptomatic liver disease, B = Hepatomegaly, C = Chronic non-cirrhotic liver disease, D = Cirrhosis, E = Decompensated cirrhosis and, F = Malignant hepatoma

— = Intermediate AAT deficiency,

- - - = Controls

two groups.

Presenting Symptoms. Twenty-nine heterozygotes and 78 controls ($p < 0.05$) were asymptomatic and investigated because of abnormal laboratory data. Hepatomegaly was present in 32 heterozygotes at debut compared to 42 controls ($p < 0.05$). Eleven heterozygotes presented with signs of advanced liver disease (Pugh groups B and C, (19)) compared to 15 controls (NS).

Laboratory Findings. The mean plasma AAT concentration in heterozygotes was 0.99 g/l compared to 1.8 g/l in controls ($p < 0.001$) and 1.35 g/l in a normal plasma pool (21). AAT values for 51% of heterozygotes and 90% of controls were above the lower normal limit of 0.9 g/l (Fig 1).

Mean serum enzyme levels were significantly elevated in both patient groups with slight differences between heterozygotes and controls (Table 2). No differences in albumin, prothrombin, and ABT score could be seen between the two groups. Mean immunoglobulin values within each subclass were similar in heterozygotes and controls. Figure 2 illustrates ABT score, ALP and immunoglobulin G levels in severity subgroups A - F. ABT score decreased succes-

Table 2. Mean Laboratory Values Among
Heterozygotes and Controls at Clinical Debut

Analysis	Ref	Heterozygotes	Controls
ASAT (ukat/l)	< 0.67	1.4	2.2
ALAT (ukat/l)	< 0.67	1.5	2.2
GT (ukat/l)	< 1.0	3.4	4.1
ALP (ukat/l)	< 4.6	6.5	6.0
Bilirubin (umol/l)	3-20	28	23
PoP (%)	> 100	83	85
ABT (%)	4-8	3.0	3.2
Albumin (g/l)	36-48	40	39
IgG (g/l)	7-15	16	15
IgA (g/l)	0.5-3	2.7	2.8
IgM (g/l)	0.4-2	1.5	1.4

sively with increased severity of liver disease in contrast to ALP and all 3 classes of immunoglobulins. There were no differences between heterozygotes and controls within each subgroup.

Autoantibodies were present in 12 heterozygotes and 24 controls. α -Fetoprotein was absent in all heterozygotes with hepatoma.

Liver Biopsy Findings. Liver biopsies were judged according to the pathologists reports according to the presence of various histological features (Table 3). Centrilobular necrosis was more frequent in controls ($p < 0.05$). Ductuli proliferation was present in 24% of heterozygotes compared to 10% of controls (NS) and steatosis was slightly more frequent among controls (NS).

The presence of PAS-positive globular inclusions after diastase treatment was not noted in any control biopsy. The presence of globules was seen in 3 biopsies from heterozygotes denied in 9 and not commented in the remaining cases.

The Cause of Liver Disease. The cause of liver disease judged from accumulated data differed significantly in the heterozygote and control subgroups ($p < 0.001$) with major differences due to

Table 3. Histological Features in Liver Biopsies from 42 Heterozygotes and 59 Controls

Features	Heterozygotes	Controls	p *
Total number of biopsies	42	59	
Cirrhosis	18	21	NS
Fibrosis	21	30	NS
Piece-meal necrosis	8	13	NS
Portal inflammation	24	38	NS
Centrilobular necrosis	19	37	< 0.05
Cholestasis	6	8	NS
Ductuli proliferation	10	6	NS
Steatosis	18	34	NS

* Calculated by χ^2 method for 2x2 contingency table with 1 d.f.

alcohol ($p < 0.01$) and cryptogenic disease ($p < 0.001$), (Table 4). Nine patients with PBC were present in the control material compared to 41 patients with PBC in the total study population (NS). One Pi SZ woman had biopsy findings compatible with PBC but lacked anti mitochondrial antibodies. The mixed group was slightly larger among heterozygotes and included 5 patients with possible cryptogenic disease and uncertain relation to moderate alcohol consumption (1 man), occupational exposure to organic solvents (2 men) or previous treatment with tuberculostatic drugs (2 men).

Clinical Course. At present 30% of heterozygotes and 41% of controls are asymptomatic (NS). Five per cent of patients in each group are currently severely ill with signs of decompensated liver cirrhosis (NS). Signs of portal hypertension with ascites, oesophageal varices and/or porto-systemic encephalopathy have developed in 19 of 64 heterozygotes and 17 of 128 controls ($p < 0.001$). Mortality has been 28% among heterozygotes and 20% among controls (NS).

The severity of liver disease based on all available data including autopsy reports in 16 of 18 deceased heterozygotes and

Table 4. Etiology of Liver Disease Among
64 Heterozygotes and 128 Controls

Subgroup	Heterozygotes	Controls	p
Alcohol	12	50	$< 0.01^*$
Vascular	7	13	NS
Systemic Disease	6	9	NS
Autoimmune	3	14	NS
Virus	2	8	NS
Mixed	20	31	NS
Cryptogenic	14	3	$< 0.001^*$
Total	64	128	$< 0.001^{**}$

* calculated by X method for 2x2 contingency table with 1 d.f.

** calculated by X method for 7x2 contingency table with 6 d.f.

22 of 26 deceased controls is summarized in Table 5. All patients with malignant hepatoma also had cirrhosis. Cirrhosis was thus present in 25 heterozygotes compared to 28 of 128 controls ($p < 0.05$).

Of the 14 heterozygotes judged to have cryptogenic liver disease, 5 are asymptomatic, 3 have hepatomegaly, 2 have chronic aggressive hepatitis, 2 have decompensated cirrhosis and 2 have died of hepatoma. One control with cryptogenic disease is asymptomatic, 1 has cirrhosis and 1 decompensated cirrhosis.

At autopsy 6 Pi MZ patients (mean age 62 yr, range 53-70) were found to have malignant hepatoma compared to one alcoholic control with hepatocellular carcinoma (61 yr). Two Pi MZ men with hemachromatosis had cholangiocellular carcinomas as did one alcoholic MZ man. An MZ man with previous intermittent clofibrate treatment had a hepatoblastoma. One man and one woman with cryptogenic disease had hepatocellular carcinomas. Malignant hepatoma was thus more frequent in intermediate AAT deficiency than among controls ($p < 0.01$). Because of the small number of cases in-

Table 5. Severity of Liver Disease at Follow Up Among 64 Heterozygotes and 128 Controls

Subgroup	Heterozygotes	Controls	p
Asymptomatic liver disease	20	56	NS
Hepatomegaly	11	29	NS
Chronic non-cirrhotic liver disease	8	15	NS
Cirrhosis (Pugh A+B)	10	16	NS
Decompensated cirrhosis (Pugh C)	9	11	NS
Hepatoma #	6	1	$< 0.01^*$
Total	64	128	$< 0.05^{**}$

*, ** = Calculated as for Table 4

= When comparing 6 hepatoma/64 heterozygotes to 13 hepatomas/793 non Pi Z liver patients, $p < 0.001$

volved, all cases of hepatoma in Malmö during the five year period were traced and 19 were found among the 857 patients in this study population, compared to 6 among the 64 heterozygotes ($p < 0.001$).

DISCUSSION

The association of liver disease with homozygous AAT deficiency is well established but few epidemiological studies on intermediate AAT deficiency and liver disease have been performed due to the difficulty of detecting heterozygotes. Their plasma AAT levels are often within the normal concentration range due to selective increases of AAT in liver disease (15) as seen in Figure 1. IEF, although reliable, is expensive and interpretation is intricate making it less suitable for large population studies. Screening for the presence of globular AAT inclusions in hepatocytes requires access to biopsy or autopsy material, is relatively insensitive in the intermediate state due to the scarcity and sporadic distribution of the globules (9), and is falsely positive in some cases of the Pi MM phenotype (22).

In this study plasma samples from 861 consecutive patients with liver disease have been screened for the presence of the Pi Z gene using a monoclonal antibody specific for the mutant domain in Pi Z AAT. This epidemiological study comprises all patients (aside from those with clear infectious disease) investigated for liver disease by liver biopsy or the ABT in Malmö during a 5 year period and thus differs from that of Fisher et al (4) who used plasma AAT levels to detect carriers of the Pi Z allele in a referral population.

Two sex and age-matched controls were selected for each heterozygote in this material. Biopsy frequency among controls and the total study population was the same (Table 1). The presence of alcohol related liver disease in 50 of 128 controls also agrees well with a previous study from this city (23), indicating that the control group is representative.

Of the heterozygotes in this report, 67% were men. This percentage differed from the total study population ($p < 0.001$) and from the study of Hodges et al (9) which includes 8 men and 14 women. The ratio of 2 males: 1 female was also found among asymptomatic Pi ZZ children with elevated serum enzyme levels (24) and in Pi ZZ adults with cirrhosis and hepatoma (3) compared to the 1:1 ratio expected from population studies (20). This discrepancy argues against a prominent role of estrogens in the development of liver disease.

Cholestasis has been a primary feature of juvenile liver disease in AAT deficiency and was reportedly present in the adult English heterozygotes with chronic active hepatitis or cryptogenic cirrhosis (9). Three of our heterozygotes had previous unexplained periods of jaundice. On the average, all heterozygotes had slightly higher bilirubin and ALP levels than controls as well as more frequent signs of ductuli proliferation on liver biopsy, but these differences were too small to be significant.

The etiology of liver disease differs notably with less alcoholism and more cryptogenic liver disease among heterozygotes than controls. We are therefore not surprised that Morin et al (5) found no increase of intermediate AAT deficiency among 132 patients with alcoholic cirrhosis. Their lack of association with cryptogenic cirrhosis is more puzzling. In our study care has been taken to include all patients with exposure to potentially hepatotoxic factors in the alcohol or mixed subgroups and the number considered cryptogenic is regarded as an underestimate.

Hodges et al (9) found no general overrepresentation of heterozygotes among patients with liver disease but found the phenotype MZ among 20% of patients with seronegative CAH and cryptogenic cirrhosis. We had few patients with these diagnoses. It is possible that Hodges et al have underestimated the presence of the Z allele, especially among asymptomatic patients with little or no inflammation in biopsies who have few large globular deposits, and have thus possibly had bias in interpreting the clin-

ical spectrum of disease. In our study, many patients with early or mild stages of liver disease have been found to have intermediate AAT deficiency and no obvious AAT inclusions in liver biopsies. Nonetheless, the severity of liver disease was generally greater in heterozygotes than controls of the same age (Table 5) and progress to severe disease (portal hypertension or malignant hepatoma) was more frequent. The prevalence of hepatoma supports the early findings of PAS-globules in 9% of patients with malignant hepatoma (2).

We conclude that intermediate AAT deficiency predisposes for chronic liver disease, constitutes a considerable subgroup of apparently cryptogenic liver disease, and increases the risk for primary liver cancer in patients with established liver disease.

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The Biosynthesis of Rat α_1 -Antitrypsin*

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The biosynthesis of rat α_1 -antitrypsin (α_1 -AT) has been studied *in vitro* in a cell-free system and in primary cultures of hepatocytes. The mRNA translation product for α_1 -antitrypsin has a 24-amino acid residue long NH₂-terminal signal peptide and its amino acid sequence was found to be (Met)-Ala-Pro-Ser-Ile-Ser-Arg-Gly-Leu-Leu-Leu-Leu-Ala-Gly-Leu-X-Cys-Leu-Ala-Pro-Ser-Phe-Leu-Ala. The signal peptide contains a central block of hydrophobic residues similar to those found for other known signal peptides. The mRNA translation product has an apparent $M_r = 43,000$. When translation was performed in the presence of dog pancreas microsomal vesicles a $M_r = 50,000$ was obtained for the segregated and core-glycosylated product. This was identical with the intracellular counterpart detected in pulse-chase experiments. Both these forms were degraded by endo- β -N-acetylglucosaminidase H and the degradations indicated that rat α_1 -antitrypsin has three oligosaccharide side chains. Terminally glycosylated endo- β -N-acetylglucosaminidase-resistant and neuraminidase-sensitive α_1 -antitrypsin was also found intracellularly prior to secretion. The M_r of this species was 52,000 whereas that of α_1 -antitrypsin synthesized in the presence of tunicamycin was 41,000, indicating an apparent $M_r = 11,000$ for the terminally glycosylated oligosaccharide side chains. The protein synthesized in the presence of tunicamycin was secreted, although more slowly than was its glycosylated counterpart, indicating that glycosylation is not a prerequisite for export of this protein.

The glycoprotein α_1 -antitrypsin is synthesized in the liver and is the major inhibitor of serine proteases in plasma (1). α_1 -Antitrypsin has been purified from the plasma of several species including man (2, 3) and rat (4-6). Most of the amino acid sequence of the human protein has been determined (7) and the complete nucleotide sequence of baboon α_1 -antitrypsin cDNA has recently been reported (8). There is a genetically determined microheterogeneity of the protein in human plasma (9). The most common phenotype called Pi MM has a normal plasma α_1 -antitrypsin concentration of approximately 1.3 g/liter. One of the more rare phenotypes, Pi ZZ, has a plasma concentration that is 10-15% of normal. This phenotype is associated with pulmonary disease and chronic

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liver disease in humans. In individuals bearing the Z allele, incompletely glycosylated α_1 -antitrypsin has been shown to accumulate in the rough endoplasmic reticulum of hepatocytes (10). α_1 -Antitrypsin is a so-called acute phase reactant with increases in its plasma concentration in response to tissue injury (1). It has also been shown to increase selectively in liver disease (11).

Agarose electrophoresis of intrahepatic α_1 -antitrypsin has revealed fractions with a more cathodal electrophoretic mobility as well as one identical with plasma α_1 -antitrypsin (12). The cathodal fractions may correspond to intercellular complexes, the presence of a propeptide analogous to that reported for albumin (13) or incompletely glycosylated forms. These observations in addition to the observed accumulation of α_1 -antitrypsin in the rough endoplasmic reticulum of hepatocytes in individuals with the Z allele initiated this study of the biosynthesis of α_1 -antitrypsin.

The present investigation reports on the biosynthesis of rat α_1 -antitrypsin both *in vitro* in a cell-free system and *in vivo* in primary hepatocyte cultures. The α_1 -antitrypsin mRNA translation product was subjected to sequence analysis. Biosynthetic modifications of the α_1 -antitrypsin carbohydrate side chains were characterized in pulse-chase experiments with and without tunicamycin and by digestion with neuraminidase and endo- β -N-acetylglucosaminidase. Our results demonstrate that rat α_1 -antitrypsin has a 24-residue prepeptide considered to be necessary for sequestration into the endoplasmic reticulum, but unlike albumin (13), it has no basic prepeptide. The *in vivo* experiments demonstrated that the intracellular α_1 -antitrypsin had been core-glycosylated and a small amount of terminally glycosylated protein could also be seen. Some of the results reported here have been published in a preliminary communication (14).

EXPERIMENTAL PROCEDURES

Materials—Pooled rat plasma was purchased from Møllegaard Farms, Skensved, Denmark. 5,5'-Dithiobis(2-nitrobenzoic acid), (Nbs)₂, N- α -p-Tosyl-L-lysine chloromethyl ketone HCl, and proteinase K were obtained from Sigma. Tritiated amino acids, L-[³⁵S]methionine, L-[³⁵S]cysteine, and L-[U-¹⁴C]asparagine of the highest available specific activities, were obtained from the Radiochemical Centre, Amersham. Oligo(dT)-cellulose (Type 2) was from Collaborative Research, Inc., ENHANCE and ¹⁴C-labeled molecular weight standards were from New England Nuclear. Guanidinium thiocyanate (purum) was from Fluka. Tunicamycin was a gift of Eli Lilly Corp. and dog pancreas microsomal vesicles prepared according to Blobel and Dobberstein (15) were obtained from Dr. Per A. Peterson, Uppsala. Agarose, blue Sepharose, DEAE-Sephadex A-50, CNBr-activated Sepharose CL-4B, and protein A-Sepharose CL-4B were purchased from Pharmacia and crystalline insulin was a gift from NOVO. Acrylamide and N,N-methylene bisacrylamide were from British Drug House and sequestrator chemicals were from Rathburn. Williams Medium E with and without methionine and fetal calf serum were purchased from Flow Laboratories. The protease inhibitors leupeptin and pepstatin A were obtained from Protein Research Foundation, Osaka. Neuraminidase (*Vibrio cholerae*) was from Behringwerke and

endo-H¹ was from Seikagaku Kogyo Co., Tokyo. All other chemicals were reagent grade.

Human serum albumin and antiserum against human serum albumin were available at the laboratory. Antiserum against rat α_1 -antitrypsin was prepared by immunization of rabbits with 200 μ g of the purified protein emulsified with 1.0 ml of Freund's complete adjuvant and one booster dose after 14 days. The immunoglobulin fraction was obtained as described (14).

Gel Electrophoresis—Agarose electrophoresis, electroimmunossay, and crossed immunoelectrophoresis were performed using standard methods (16–18). SDS-PAGE was performed in 10% polyacrylamide gels according to Laemmli (19). Slab gels with a 10–15% polyacrylamide gradient, 20 $\times$ 30 cm with a 2-cm spacing gel, were run at 25 mA constant current for 16 h according to Blobel and Dobberstein (20) using the gel system of Maizel (21). Gels with radioactive proteins were dried after fixation and treatment with ENHANCE according to the manufacturer's instructions and thereafter exposed for 3 to 10 days to rapid processing x-ray film at -70°C before developing.

Purification of Rat Plasma α_1 -Antitrypsin—Rat plasma α_1 -antitrypsin was isolated from 380 ml of pooled rat plasma containing 0.015 M potassium oxalate using thiol-disulfide interchange chromatography as the first step (2, 3). The eluate was dialyzed extensively against 0.05 M Tris HCl, 0.15 M NaCl, pH 8.0, at $4-8^\circ\text{C}$ to remove excess 5,5'-dithiobis(2-nitrobenzoic acid). The major contaminant in the dialyzed eluate was albumin as judged by agarose electrophoresis. It was removed by chromatography at room temperature on a blue Sepharose column (14 $\times$ 2.2 cm) equilibrated with the above buffer, flow rate 20 ml/h. Fractions of the eluate containing α_1 -antitrypsin, as judged by agarose gel electrophoresis and immunoelectrophoresis, were pooled and concentrated by precipitation with 75% ammonium sulfate. The precipitate was collected by centrifugation, dissolved in the same buffer, and further purified by chromatography on Ultrogel AcA 44 (100 $\times$ 2.6 cm) at $4-8^\circ\text{C}$. The flow rate was 13.3 ml/h and 20-min fractions were collected. The fractions containing α_1 -antitrypsin were pooled and stored at -20°C .

Amino Acid Analysis and NH_2 -terminal Sequence Determination—The amino acid composition of α_1 -antitrypsin was determined after 24, 48, and 72 h of hydrolysis of 0.25 mg of reduced and S-carboxymethylated protein samples in 6 M HCl with norleucine as internal standard using a Beckman 119 CL amino acid analyzer (22). Values for serine and threonine were extrapolated to zero hydrolysis time. Tryptophan was determined spectrophotometrically according to Edelhoch (23). The amino acid sequence of the rat plasma protein was determined by automated Edman degradation (24) in a Beckman 890 C amino acid sequencer using the Beckman 1 M Quadrol program. 2.3 mg of carboxymethylated lyophilized protein were dissolved in 400 μ l of formic acid containing 2 mg of Polybrene and applied to the sequencer cup. The phenylthiohydantoin derivatives were identified by HPLC as previously described (25).

Messenger RNA Preparation and Cell-free Translation—Messenger RNA was isolated from livers of Sprague-Dawley rats treated 24 h prior to hepatectomy with subcutaneous injections of 0.5 ml of turpentine/100 g of body weight to induce an inflammatory response. The method of Chirgwin *et al.* (26) was used with 50% instead of 75% ethanol in the first precipitation step and oligo(dT)-cellulose chromatography as described. RNA was quantitated using an A_{260} at 260 nm of 200.

In vitro translation of mRNA was performed in a staphylococcal nuclease-treated rabbit reticulocyte lysate prepared according to Pelham and Jackson (27). Incubations were for 90 min at 35°C in the presence of 19 unlabeled and one radioactively labeled amino acid. In some experiments, nuclease-treated dog pancreas microsomal vesicles were present at a final concentration of 3.5 A_{260} units/ml. Total incorporation of radioactivity into protein was estimated by measurement in a Packard Tri-Carb liquid scintillation counter after trichloroacetic acid precipitation of a small aliquot of the incubate on filter paper. Immunoprecipitation of the α_1 -antitrypsin translation product was performed in 1% Nonidet P-40 buffer according to Dobberstein *et al.* (28), using 2 μ l of normal rabbit serum and 1 μ l of anti-rat α_1 -antitrypsin/25 μ l of incubation mixture and protein A-Sepharose. After extensive washing, α_1 -antitrypsin was eluted from the protein A-Sepharose with 1% SDS. Eluted proteins were either visualized on SDS-PAGE slabs with fluorographic enhancement (29) or subjected

to amino acid sequencing. For sequence determinations, large scale cell-free translations were performed resulting in 0.5–5 ng of immunoprecipitated α_1 -antitrypsin which was eluted in 2–4 ml of 1% SDS. Sperm whale myoglobin (300 nmol or 50 nmol when HPLC was to be used for identification) was added as a carrier protein prior to precipitation by the addition of 5 volumes of acetone, 0.1 M in HCl. Following incubation overnight at room temperature and centrifugation at 4°C , 10,000 rpm for 30 min in a Sorvall HB-4 swing-out rotor, the pellet was dissolved in 400 μ l of formic acid and applied to the sequencer cup. The Beckman 1 M Quadrol program was used. After drying, one wash cycle omitting heptafluorobutyric acid was performed. The anilinothiozolinones were dried under N_2 , dissolved in 200 μ l of methanol, and the radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. When HPLC was used for identification, carrier phenylthiohydantoin-amino acids were included together with norleucine (25 nmol of each) in the sequencer collecting tubes. The anilinothiozolinones were dried under N_2 and converted to phenylthiohydantoin derivatives using standard procedures (24). The residues were dissolved in 20 μ l of ethyl acetate, injected manually into the HPLC column and separated as previously described. Fractions corresponding to the labeled amino acids were collected manually in scintillation vials and their radioactivity was measured.

Labeling of Rat Hepatocytes—Hepatocytes were isolated from normal Sprague-Dawley rats weighing 150–280 g by the collagenase perfusion method of Seglen (30) using the modifications of Åkesson (31). To Williams Medium E (with and without methionine) were added the following substances: glutamine, 300 mg/liter; insulin, 0.5 mg/liter; benzylpenicillin sodium, 100 mg/liter; streptomycin, 50 mg/liter; and dexamethasone, 4 mg/liter. Cells were filtered through a nylon net and allowed to sediment for 10 min in William's Medium E. They were then counted and the yield was 200–400 $\times 10^6$ cells/liver. Fewer than 10% of the cells were stained by trypan blue. Cell culture was performed in 50-mm plastic Petri dishes, pretreated with 0.2 mg of collagen/dish. Each dish contained 4×10^6 cells in 2 ml of medium with 10% fetal calf serum and the incubation was performed in humidified air with 5% CO_2 at 37°C for 1 h. Free cells and medium were removed and fresh medium with 10% fetal calf serum was added to the cell monolayers and the incubation continued for 20 h. At this time, the cell monolayers were washed twice in William's Medium E lacking methionine and preincubated in this medium without fetal calf serum for 75 min. Tunicamycin in an aqueous solution, pH adjusted to 11–12 with NaOH, was included at a concentration of 3 $\mu\text{g}/\text{ml}$ in the preincubation medium of appropriate dishes. The preincubation was terminated by the addition of 130 μCi of [^{35}S]methionine (1300 Ci/mmol) to each dish and incubation was continued for a 15-min pulse. The cell monolayers were then washed once in the chase medium, which contained five times the normal concentration of unlabeled methionine, and incubated in this medium until harvesting. At appropriate intervals, media were removed and immediately frozen in liquid nitrogen. Cells were lysed in 0.4 ml of 1% Nonidet P-40 containing 0.15 M NaCl, 3.75 mg/liter of *N*- α -tosyl-L-lysine chloromethyl ketone HCl, 10 mg/liter of leupeptin, 10 mg/liter of pepstatin, 0.001 M phenylmethylsulfonyl fluoride, 0.010 M Tris-HCl, pH 7.5 (lysing buffer), and were immediately frozen in liquid nitrogen. Cells and media were maintained at -70°C until use.

Thawed media were spun in an Eppendorf centrifuge for 5 min and immunoprecipitation was performed on the supernatant as for translation products. Cell lysates were thawed, spun as above, and further centrifuged in a Beckman Airfuge at 30 p.s.i. (132,000 $\times g$) for 10 min. Human serum albumin was added to supernatants to a final concentration of 5 mg/ml (32). After the addition of 25 μ l of rabbit anti-human serum albumin/0.4 ml of lysate, the mixture was incubated overnight at 8°C followed by addition of 300 μ l of protein A-Sepharose slurry and incubation with shaking for 2 h at room temperature. The supernatant after centrifugation was subjected to specific immunoprecipitation as for the translation mixtures.

Digestion with Neuraminidase and Endo- β -acetylglucosaminidase—Aliquots from mRNA translations in the presence of microsomal vesicles (50 μ l + 200 μ l of lysis buffer), rat liver cell lysates (250 μ l), or cell media (500 μ l with protease inhibitors as described above) were mixed with equal volumes of 0.1 M sodium acetate, pH 5.5, containing 0.3 M NaCl, 0.01 M CaCl_2 . To each sample were added 20 μ l of neuraminidase (1 unit/ml) and incubation was performed at 37°C with further additions of 20 μ l of enzyme at 30, 60, and 120 min. The incubation continued for 120 min after the last enzyme addition and was terminated by the adjustment of pH to 8.0 by the addition of 2.0 M Tris-HCl, pH 8.0. Thereafter, immunoprecipitation was performed using human serum albumin as described.

¹ The abbreviations used are: endo-H, endo- β -N-acetylglucosaminidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

Endo-H was dissolved in 0.3 M citrate buffer, pH 5.5, at a concentration of 1 unit/ml and stored at -70°C . After immunoprecipitation of α_1 -antitrypsin from cell lysates, media, and translation incubates, the protein was eluted from the protein A-Sepharose with 75 μl of 0.05 M Tris-HCl, pH 6.8, containing 2% SDS. The total radioactivity in the eluates was calculated by radioactivity measurements of 5- μl aliquots. Aliquots (30 μl) from each eluate including one from the medium as a blank were heated for 3 min at 100°C . To each sample were added 20 μl of endo-H solution (20 μl of citrate buffer to the medium blank) and the samples were incubated for 20 h at 37°C . To each sample were then added 50 μg of bovine serum albumin as carrier protein and the proteins were precipitated with 20% trichloroacetic acid for 2 h on ice. The samples were centrifuged and pellets were washed once with absolute ethanol and once with ether, dried, and dissolved in the sample preparation solution for SDS-PAGE.

RESULTS

Purification and Characterization of Rat Plasma α_1 -antitrypsin—Thiol-disulfide interchange was used as the first step in the purification of α_1 -antitrypsin (2, 3). The overall recovery was 16% (Table I) which is comparable to the yields reported by two authors who used conventional procedures (4, 5). Our method requires less work than the previously reported methods.

The purified plasma protein migrated as a single band on SDS-PAGE rods with an apparent $M_r = 52,000$ (14). This may be compared with the $M_r = 50,000$ reported by Takahara *et al.* (4) (sedimentation analysis) and 47,000 reported by Roll and Glew (5). The amino acid compositions of α_1 -antitrypsin purified with the three different methods are in good agreement (Table II). Serum from rabbits immunized with the purified rat α_1 -antitrypsin gave a single peak on crossed immunoelectrophoresis of total rat plasma in dilutions in 1:25 to 1:200 using 5–15% antiserum in the agarose gel. The isolated protein was titrated by added trypsin and chymotrypsin and quantitative complexation was indicated by agarose electrophoresis. The purity of the α_1 -antitrypsin was further substantiated by automated Edman degradations which enabled unambiguous identification of the 17 NH_2 -terminal residues except for the residue in position 14 (Fig. 1). The sequence is similar to those of the baboon (8) and human (7) α_1 -antitrypsin with identical residues in the three proteins in positions 1, 2, 4, and 12, and Pro at position 15 in rat and baboon α_1 -antitrypsin. These findings differ from those of Roll and Glew (5) who reported Ala as the NH_2 -terminal amino acid using the 5-dimethylaminonaphthalene-1-sulfonyl chloride method.

Cell-free Translations of Rat Liver mRNA in the Presence and Absence of Microsomal Vesicles—Poly(A)-RNA was ob-

tained from rat liver after chromatography on oligo(dT)-cellulose with a yield of approximately 0.3–1.2 mg/g of liver, wet weight. The A_{260}/A_{280} quotient for mRNA was always greater than 2.07. On incubation of the mRNA in the reticulocyte lysate system in the presence of [^{35}S]methionine, 10–25% of added radioactivity was incorporated into trichloroacetic acid-precipitable protein with maximal incorporation at mRNA concentrations of approximately 40 $\mu\text{g}/\text{ml}$. Immunoprecipitable α_1 -antitrypsin accounted for 0.5–2% of the trichloroacetic acid-precipitable radioactivity.

Slab gel electrophoresis of total rat liver mRNA translation products is shown in Fig. 2, lane 1. The immunoprecipitated α_1 -antitrypsin (lane 3) had an apparent $M_r = 43,200$. The intensity of the immunoprecipitated α_1 -antitrypsin band decreased on the addition of increasing amounts of the unlabeled plasma protein, confirming the immunoreactive identity of the radioactively labeled protein with rat plasma α_1 -antitrypsin. Identical results were obtained in one experiment using specific antibodies against rat plasma α_1 -antitrypsin prepared by immunosorbent chromatography of the antiserum on a column of insolubilized rat plasma α_1 -antitrypsin.

Most eukaryotic secretory and plasma membrane proteins are translated with hydrophobic NH_2 -terminal sequences that serve as signals for translocation across the endoplasmic reticulum membrane (33). Microsomal vesicles from dog pancreas, if added prior to mRNA in the cell-free translation system, will remove the signal sequences and core-glycosylate appropriate residues (15, 20). Comparisons of products obtained on translation in the presence and absence of microsomal vesicles

TABLE II

Amino acid composition of rat α_1 -antitrypsin calculated for an apoprotein $M_r = 41,000$

All values unless otherwise specified are averages of one measurement after 24, 48, and 72 h of hydrolysis. Values reported by other authors are recalculated to an apoprotein $M_r = 41,000$ for comparison.

Amino acid	Present study	Takahara ^a	Roll ^c
Lys	21.9	26.4	26
His	10.0	10.2	9
Arg	12.6	11.5	12
Asp	43.2	47.9	42
Thr ^b	25.8	28.9	28
Ser ^b	30.8	28.0	29
Glu	38.7	44.0	37
Pro	17.1	20.9	16
Gly	16.6	18.8	19
Ala	20.8	24.4	23
Cys ^c	1.3	1.9	2
Val ^d	19.5	21.6	23
Met	11.0	11.0	10
Ile ^d	19.1	16.9	14
Leu	39.2	45.1	40
Tyr	9.0	8.0	8
Phe	22.5	23.2	22
Trp ^e	3.3	3.0	4

^a Takahara *et al.* (4) and Roll and Glew (5).

^b Extrapolated to zero hydrolysis time.

^c Determined as S-carboxymethyl cysteine.

^d Value at 72 h of hydrolysis time.

^e Determined spectrophotometrically according to Edelhoch (23).

TABLE I
Purification of rat α_1 -antitrypsin

Step	Vol- ume	Total protein	α_1 -AT	Purifi- cation factor	Yield
	ml	mg	arbitrary units		%
Plasma	380	24,300	26,600	1.0	100
Thiol-disulfide inter- change	86	483	8,020	15	30
Blue Sepharose	104	294	7,490	23	28
Ultrogel AcA 44	54	151	4,290	26	16

FIG. 1. The NH_2 -terminal sequence of plasma α_1 -antitrypsin from the rat determined by automated Edman degradations. The baboon sequence, deduced from the cDNA sequence (8), and the sequence of human α_1 -antitrypsin determined by conventional protein sequencing (7) are shown for comparison.

	1	10
Rat γ_1 -antitrypsin	Glu-Asp-Ala-Gln-Glu-Arg-Asp-Arg-His-Gln-Gln-Asp-Gln-?-Pro-Thr-Tyr-	
Baboon γ_1 -antitrypsin	Glu-Asp-Pro-Gln-Gly-Asp-Ala-Ala-Gln-Lys-Thr-Asp-Thr-Pro-Pro-His-Asp-	
Human γ_1 -antitrypsin	Glu-Asp-Pro-Gln-Gly-Asp-Ala-Ala-Gln-Lys-Thr-Asp-Thr-Ser-His-His-Asp-	

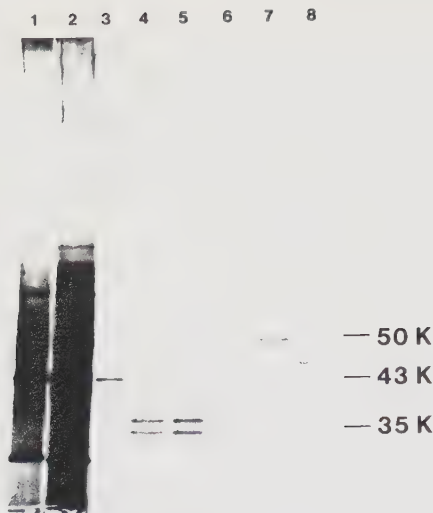


Fig. 2. α_1 -Antitrypsin synthesized *in vitro* in the absence and presence of dog pancreas microsomal vesicles. SDS-PAGE 10–15% gradient slab gel with fluorographic enhancement showing the [35 S]methionine-labeled total rat liver mRNA translation products in the absence (lane 1) and presence (lane 2) of microsomal vesicles. In the absence of vesicles, the immunoprecipitated mRNA translation product for α_1 -antitrypsin had a $M_r = 43,200$ (lane 3). This product after incubation with proteinase K in the absence (lane 4) and presence (lane 5) of 0.25% Nonidet P-40 demonstrates two peptides of $M_r = 37,300$ and $35,500$. Lanes 6–8 show the corresponding products of synthesis in the presence of microsomal vesicles ($M_r = 50,200$, lane 6) after the addition of proteinase K (lane 7) and proteinase K plus 0.25% NP-40 (lane 8, $M_r \approx 47,000$ and $45,200$).

are shown in Fig. 2. In the absence of vesicles, the α_1 -antitrypsin translation product with $M_r = 43,200$ (lane 3) was cleaved on the addition of proteinase K in two steps giving products with $M_r = 37,300$ and $35,500$. The reaction was the same in the presence and absence of detergent (lanes 4 and 5). In the presence of vesicles, the M_r of the α_1 -antitrypsin translation became $50,200$ (lane 6). In the absence of detergent, intact vesicles protected the translation products from proteolytic digestion, indicating translocation across the membranes (lane 7). On the other hand, addition of proteinase K after disruption of the membranes by 0.25% Nonidet P-40 allowed digestion to products of $M_r = 47,000$ and $45,200$ (lane 8). All molecular weights given are averages of two experiments in close agreement. The difference of $1,800$ in M_r of the cleavage products with and without vesicles indicates cleavage at identical points along the polypeptide chain.

Thus, the apparent molecular weight of the carbohydrate moiety should correspond to the difference of $45,200$ minus $35,500$ or $9,700$. The peptide removed by the first cleavage in the standard translation system ($43,200$ minus $37,300$ or $5,900$) is larger than that removed in the microsomal vesicle system ($50,200$ minus $47,000$ or $2,800$), suggesting that the cleavage sites for proteinase K are located near the NH_2 -terminal end of the polypeptides. No evidence exists to suggest removal of a glycosylated peptide in the proteinase K cleavage as involvement of such peptides would result in larger fragments removed from the vesicle system than from the product of the standard translation system. The apparent M_r values calculated for the carbohydrate moiety and the vesicle translation product agree well with the results of *in vivo* experiments (see below).

In Vivo Synthesis and Glycosylation of Rat α_1 -Antitrypsin—Interpretation of the above data is complicated by the simultaneous cleavage of signal sequences and core glycosylation achieved by the microsomal vesicles. Core glycosylation can be prevented *in vivo* by the antibiotic tunicamycin which blocks the addition of *N*-acetylglucosamine to dolichol phosphate (34), the first step in the formation of the core oligosaccharides. Pulse-chase experiments using isolated rat hepatocytes gave the following additional information (Fig. 3). At the beginning of the chase period (0 min, lane 1), all α_1 -antitrypsin radioactivity was present intracellularly with a $M_r = 50,000$, corresponding well to the $M_r = 50,200$ found for the vesicle translation product. At this time, no radioactivity could be immunoprecipitated from the medium (lane 11). After preincubation of the cells with tunicamycin, the intracellular protein had a $M_r = 41,000$ (lanes 2, 4, 7, and 10), indicating a $M_r = 9,000$ for the core oligosaccharide, in agreement with the above calculations. At 15 min after termination of the pulse, a very weak band with $M_r = 52,000$ was discernible in the cell lysate in addition to the $50,000$ band. This band had slightly greater intensity after 30 and 45 min (lanes 5 and 6). Thereafter, the $50,200$ band became weaker and disappeared more rapidly than the $52,000$ band (lanes 8 and 9 at 60 and 90 min, respectively). An immunoreactive band of the same size as the larger intracellular band appeared extremely weakly in the medium at 15 min, increasing in intensity with time (lanes 12–16) and should thus represent the mature plasma protein. This increase in approximately $M_r = 2,000$ should correspond to the terminal glycosylation step, *i.e.* trimming of terminal hexose residues and the addition of *N*-acetylglucosamine, Gal, and sialic acid residues found in rat plasma α_1 -antitrypsin (6). The time intervals between translation, sialylation, and secretion cannot be exactly determined from this study, but the mature protein begins to appear in the medium 30 min after initiation of the radioactive pulse. The terminal glycosylation was totally inhibited by tunicamycin as expected. On the other hand, glycosylation was apparently not a prerequisite for secretion as rat α_1 -antitrypsin with a $M_r = 41,000$ corresponding to the translation product minus signal peptide did appear in the medium of the tunicamycin-treated cells (lanes 17 and 18). The peak of intracellular α_1 -antitrypsin radioactivity as well as the appearance of the polypeptide in the medium were delayed by 15–30 min in the tunicamycin-treated cells compared to the untreated cell cultures.

To further characterize the various bands, labeled rat α_1 -antitrypsin was incubated with endo-H or neuraminidase. The enzyme endo-H cleaves the glycosidic bond between the core *N*-acetylglucosamines of the Asn-linked oligosaccharides and is specific for glycoproteins in the "high mannose" form, *i.e.* the form present prior to terminal glycosylation in the Golgi apparatus (35). On the other hand, neuraminidase reacts only with the final terminal sialic acid residues. Cell lysates, medium, and the products of translation in the presence of microsomal vesicles were subjected to digestion by these enzymes. The results are shown in Fig. 4. Here, the mRNA translation product in the absence of microsomal vesicles (lane 1) was only slightly larger than the intracellular protein synthesized in the presence of tunicamycin ($43,200$ and $41,000$, respectively). The translation product in the presence of vesicles ($M_r = 50,200$, lane 2) was not affected by neuraminidase digestion (lane 3), but disappeared after digestion with endo-H (lane 4). Three bands with $M_r = 42,000$, $45,000$, and $47,000$ appeared. The presence of three bands indicates that the digestion was not complete and the staggered appearance suggests that the products differ by one oligosaccharide chain each. This would agree with three oligosaccharide chains in analogy to results reported for the human protein (7).

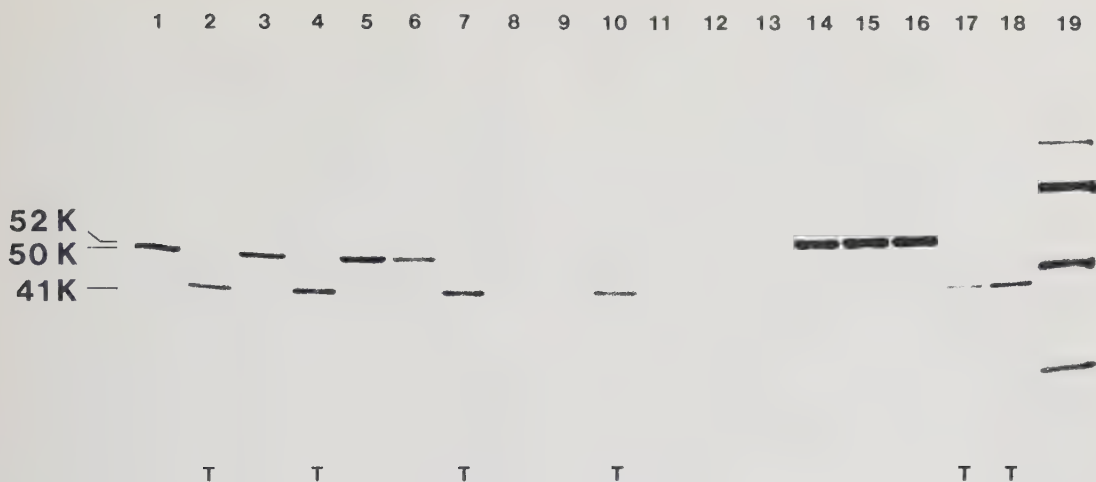


FIG. 3. Rat α_1 -antitrypsin synthesized *in vivo* in a pulse-chase experiment. Isolated rat hepatocytes were pre-incubated for 75 min in methionine-free medium with tunicamycin, 3 μ g/ml, in lanes marked T. After a 15-min pulse with [35 S]methionine, chase was performed in a medium with a high concentration of unlabeled methionine. SDS-PAGE in a 10–15% gradient slab gel with fluorographic enhancement is shown. Immunoprecipitated protein from cell

lysates is shown at chase times 0 (lanes 1 and 2), 15 (lanes 3 and 4), 30 (lane 5), 45 (lanes 6 and 7), 60 (lane 8), and 90 min. Lanes 11–16 show immunoprecipitated α_1 -antitrypsin from the medium of cells not treated with tunicamycin at chase periods 0, 15, 30, 45, 60, and 90 min. Immunoprecipitates from the medium of tunicamycin-pretreated cells at 45 and 90 min of chase time are shown in lanes 17 and 18, respectively.



FIG. 4. Neuraminidase and endo-H treatment of rat α_1 -antitrypsin synthesized in cell-free and hepatocyte incubations on 10–15% SDS-PAGE with fluorographic enhancement. *In vitro* mRNA translation was performed as in Fig. 4 with the untreated immunoprecipitated translation product shown in lane 1. Aliquots containing the α_1 -antitrypsin product of translation in the presence of microsomal vesicles (lane 2) were treated with neuraminidase (lane 3) and endo-H (lane 4). (The bands in lanes 2 and 3 have the same

mobility but are weaker in lane 3 because of sample loss.) Rat hepatocyte lysate after 45-min chase is shown in lane 5, after neuraminidase (lane 6) and endo-H digestion (lane 7). Aliquots of medium after 90-min chase are likewise untreated (lane 10), incubated with neuraminidase (lane 11), endo-H (lane 12), or incubated with the endo-H buffer as a blank (lane 13). Lanes 8 and 9 show intracellular α_1 -antitrypsin synthesized after pre-incubation with tunicamycin at 30 and 45 min of chase time.

When the intracellular α_1 -antitrypsin protein species (45 min of chase time, lane 5) were treated with neuraminidase, only the 52,000 band disappeared. A band of similar intensity appeared immediately above the 50,200 band which remained unchanged (lane 6). Thus, the 52,000 band apparently contained sialic acid, unlike its 50,200 precursor. In contrast, endo-H digestion had no effect on the 52,000 band, but gave rise to bands at 42,000, 45,000, and 47,000 upon disappearance of the 50,200 species (lane 7). In these respects, the 50,200 *in vivo* product appeared identical with the product of *in vitro* mRNA translation in the presence of microsomal vesicles. The smallest endo-H degradation product in both cases was only slightly larger than the α_1 -antitrypsin produced by cells pre-incubated with tunicamycin (lanes 8 and 9), as expected, since endo-H removes all carbohydrate but one N-acetylglucosamine residue from each oligosaccharide chain. The protein secreted from isolated hepatocytes into the medium is shown in lane 10 and reacted similarly to the 52,000 intracellular band (lane 5) on neuraminidase digestion (lane 11). Digestion with endo-H (lane 12) or incubation in the citrate buffer (lane 13) had no effect on the exported protein which is terminally glycosylated, excluding the possibility that proteolytic activity in the enzyme was responsible for the apparent decrease in *M_r* seen in the other incubations.

The NH₂-terminal Amino Acid Sequence of Rat Pre- α_1 -Antitrypsin—The data presented above and deduced from Figs. 2 and 3 indicate the presence of a signal peptide with a *M_r* ~2,000 in the mRNA translation product for α_1 -antitrypsin.

In order to characterize this putative peptide, mRNA translations were performed in the presence of each of 20 radioactively labeled amino acids separately. The liquid scintillation measurements made on the products of automated Edman degradation or on the manually collected fractions obtained from HPLC are shown in Fig. 5 for the amino acids where positive identifications could be made. Positions -23 to -10 could be filled by amino acids having more than twice the counts/min in the step in question as in the immediately preceding step. The amount of radioactivity in positions -7, -4, -2, -1, 2, 3, and 7, although not fulfilling the above criterion, was consistent with the repetitive yield of the degradations. This criterion could only be applied to degradations in which the residue in question was found at least twice. At positions -8 and -3, assigned to Cys and Phe, relatively low peaks were accepted in the absence of any significant radioactivity at these positions from any other degradations. No assignment could be made at -9. No Asn, His, Lys, Met, Thr, Trp, Tyr, or Val residues could be identified in the first 30 NH₂-terminal positions.

Although 900 cpm were measured in position -23 for [³⁵S] methionine, the blank sequencing cycle for that degradation contained 3,700 cpm. As the blank cycle for Ala contained only 4 compared to 350 cpm found in step 1, and as these 350 cpm were consistent with the 96% repetitive yield calculated for the other Ala residues, Ala was assigned to this position. It is known that mRNA translation products always begin with a Met residue. However, initial Met residues followed

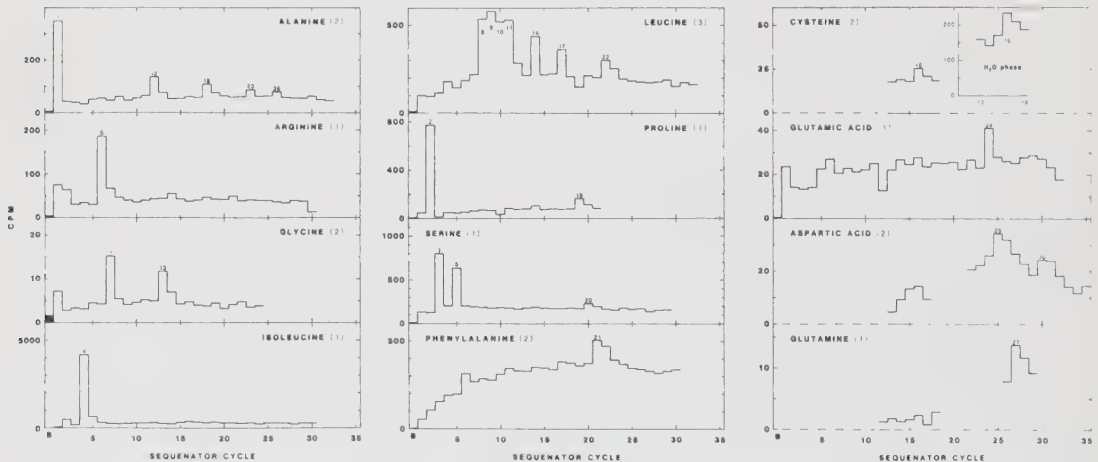


Fig. 5. Sequential Edman degradation of rat α_1 -antitrypsin synthesized in a reticulocyte lysate with different labeled amino acids. Results for Ala, Gly, Leu, Cys, Glu, Asp, and Gln are after separation by HPLC. Numbers in parentheses indicate the number of degradations performed on separate *in vitro* translations and the cycle numbers above the histograms indicate positive identification in the sequence of rat pre- α_1 -antitrypsin, numbered according to sequenator cycle. B is the blank sequenator cycle without heptafluorobutyric acid.

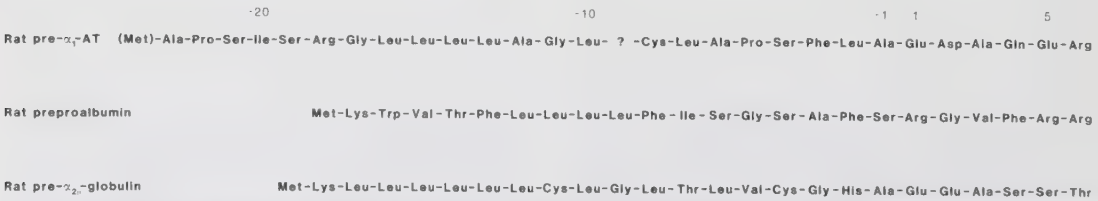


Fig. 6. Amino acid sequences of signal peptides for the known rat liver secretory proteins. The residues are numbered in a retrograde direction from the point of attachment to the plasma protein for α_1 -antitrypsin and α_2 -globulin (38) or to the propeptide for albumin (14). The methionine in parentheses represents the initiator methionine removed during synthesis in the reticulocyte lysate.

immediately by small uncharged amino acids, in particular Ala, are frequently removed by an aminopeptidase known to be present in the reticulocyte lysate (36).

By identification of Glu at sequencing cycle 24, Asp at 25 and 30, Ala at 26, and Gln at 27 and by alignment with the NH₂-terminal sequence of the plasma protein (compare to Fig. 1), a length of 24 amino acid residues (including Met at -24) for the signal peptide was clearly established. The final amino acid sequence for the signal peptide is shown together with the other known signal sequences for rat liver secretory proteins in Fig. 6. Assuming a residual weight of 100 at position 15, a $M_r = 2,398$ can be calculated for the 24-amino acid signal peptide.

DISCUSSION

Intracellular forms of various plasma proteins have been identified in the liver and early studies of intracellular precursors in hepatocytes have utilized methods of extraction and chromatographic separation or immunoelectrophoresis (12, 37, 38). Using these methods together with injection of radioactively labeled amino acids in rats, perfused livers, slices, or cell cultures, it has been possible to identify specific precursor forms (37-40). Cell-free *in vitro* synthesis of rat liver proteins has been performed in a few cases and has demonstrated the presence of transient NH₂-terminal signal peptides in the translation products (13, 39, 41). In the case of albumin, a nonglycosylated protein, an additional hexapeptide has been found at the NH₂ terminus of the intracellular protein after cleavage of the signal peptide. This so-called propeptide contains three basic residues with a terminal sequence Arg-Arg-, allowing specific enzymic recognition for cleavage (13). Recently, intracellular α_1 -acid glycoprotein has been reported to have the same NH₂-terminal amino acid sequence as the mature plasma protein (40). The NH₂-terminal amino acid sequence of rat α_{2u} -globulin, a nonglycosylated protein, has also recently been deduced from cDNA sequencing combined with protein sequencing of the *in vitro* translation product and shown to contain a signal peptide but no basic propeptide (39).

In this study, we have combined the techniques of *in vitro* mRNA translation, including the addition of dog pancreas microsomal vesicles, and *in vivo* pulse-chase experiments in primary hepatocyte cultures to further elucidate the biosynthesis and secretion of rat plasma α_1 -antitrypsin. We have determined the NH₂-terminal sequence of the mRNA translation product and found a signal peptide of 24 amino acid residues. The microsequencing extended well into the first residues of the mature plasma protein. The NH₂-terminal extension of the *in vitro*-synthesized α_1 -antitrypsin had all the characteristic features found in most previously sequenced signal peptides: 1) a small uncharged residue at the site of cleavage, *i.e.* alanine, serine, or glycine; 2) a basic residue at position -17 or -18; 3) a hydrophobic sequence after the basic residue; and 4) a glycine or proline residue that interrupts the hydrophobic sequence (39). It can be concluded that rat α_1 -antitrypsin, in similarity to α_{2u} -globulin and α_1 -acid glycoprotein, lacks the basic propeptide found in albumin. Comparison of the sequences of the three signal peptides for rat liver proteins known to us (Fig. 6) does not disclose any obvious structural properties unique to these peptides, which is interesting as all three proteins are presumably synthesized by the same hepatocytes (42). We did not identify the initiator methionine residue at position -24 in pre- α_1 -antitrypsin. It has, however, been unequivocally shown that the reticulocyte lysate contains an aminopeptidase that cleaves the initiator methionine residue when it is followed by an alanine residue (36).

α_1 -Antitrypsin was synthesized in the presence of dog pancreatic microsomal vesicles, involving translocation across the vesicle membrane, cleavage of the signal peptide, and core glycosylation. The difference in M_r of the translation product in the presence and absence of vesicles was 7,000. Furthermore, differences in proteolytic cleavage fragments of these two products indicated an apparent $M_r = 9,700$ for the carbohydrate prosthetic group of the core-glycosylated protein. As the peptide removed by the first proteolytic cleavage was larger in the absence than in the presence of microsomal vesicles, the cleavage can be deduced to occur near the NH₂-terminal end of the proteins, removing even the signal peptide from the primary translation product. From this experiment, the M_r of the signal peptide would be 9,700 minus 7,000 or 2,700. The difference in apparent molecular weights of the primary translation product and the intracellular α_1 -antitrypsin synthesized in the presence of tunicamycin was 2,200. These values are in good agreement with the calculated value of approximately 2,400 for the 24-amino acid signal peptide from sequence determinations.

In the pulse-chase experiments, α_1 -antitrypsin was first discernible in the medium 30 min after initiation of the radioactive pulse. Unglycosylated α_1 -antitrypsin synthesized in the presence of tunicamycin was also released into the medium although somewhat more slowly. The endo- β -*N*-acetylglucosaminidase H digestions showed that the $M_r = 50,200$ dominating intracellular fraction of α_1 -antitrypsin was core-glycosylated and the appearance of two discrete intermediate bands during digestion suggested that rat α_1 -antitrypsin, like its human counterpart (7), has three oligosaccharide side chains. Prior to the appearance of mature α_1 -antitrypsin in the medium, a component with the same mobility was visible intracellularly. This intracellular α_1 -antitrypsin as well as that secreted into the medium, was resistant to digestion by endo-H but susceptible to neuraminidase, indicating that it had been terminally glycosylated, presumably in the Golgi apparatus. The apparent molecular weight of the carbohydrate prosthetic group in the terminally glycosylated protein was 11,000 based on a comparison with the protein synthesized in the presence of tunicamycin. Demonstration of the core-glycosylated α_1 -antitrypsin intracellularly in the rat as well as the demonstration of intracellular precursors for α_1 -antitrypsin and α_1 -acid glycoprotein on DEAE-cellulose chromatography (38) suggest that one of the extracellular peaks seen on crossed immunoelectrophoresis of human liver extracts (12) corresponds to the core-glycosylated species.

In humans with the Z allele for α_1 -antitrypsin deficiency, globules of periodic acid-Schiff-stainable α_1 -antitrypsin immunoreactive material are found in the rough endoplasmic reticulum. Carbohydrate analysis of this material suggests that it is at least partly core-glycosylated protein in the high mannose form (43). The human Z α_1 -antitrypsin protein is known to contain a Glu→Lys substitution compared to the M protein (44) and processing through the export channels of the cell may be inhibited by some mechanism related to solubility. In this content, it is interesting to note that rat α_1 -antitrypsin synthesized in the presence of tunicamycin and thus totally lacking in carbohydrate is secreted from hepatocytes with only a slight delay compared to the glycosylated protein. This result may be viewed in light of a recent report on the differing requirements for glycosylation in the secretion of immunoglobulins of various classes. In a hybridoma cell culture producing secretory IgM and IgG, tunicamycin nearly prevented the secretion of IgM but did not interfere with the secretion of IgG (45, 46). This suggested that secretion from the cell was neither dependent on the degree of glycosylation nor on characteristics of the cell, but rather was probably

dependent on characteristics inherent in the primary structure of the protein in question.

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The Biosynthesis of Abnormally Glycosylated Alpha₁-Antitrypsin in a Human Hepatoma Cell Line

by

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The human hepatoma cell line PLC/PRF/5 synthesized and secreted a functional alpha₁-antitrypsin glycoprotein with normal molecular size but retarded electrophoretic mobility. The total process of translation, glycosylation and export required about 40 min and followed the same synthetic pattern as seen in rat hepatocytes i.e. a signal peptide is cleaved cotranslationally, a core-glycosylated protein in the high-mannose form is formed in the rough endoplasmic reticulum, trimmed, and a stable complex-glycosylated alpha₁-antitrypsin is found intracellularly prior to export. Alpha₁-antitrypsin export to medium was delayed by tunicamycin, inhibited by cycloheximide but unaffected by colchicine. After addition of exogenous alpha₁-antitrypsin to culture medium neither negative nor positive feedback induction of synthesis could be demonstrated.

Electrophoretic techniques indicated the presence of atypical, highly branched but incompletely sialylated carbohydrate chains in the hepatoma cell derived alpha₁-antitrypsin. The accumulation of intracellular alpha₁-antitrypsin inclusions seen in the endoplasmic reticulum may reflect an imbalance between a high rate of polypeptide synthesis and terminal glycosylation.

ABBREVIATIONS: α_1 -AT = α_1 -Antitrypsin, endo-H = endo- β -N-acetylglucosaminidase, HC = Hepatocellular carcinoma, M_r = Apparent molecular radius, M-FCS = medium + fetal calf serum, PAS = periodic acid Schiff, PBS = phosphate buffer saline, Pi = Protease inhibitor, SFM = Serum free medium, SDS-PAGE = sodium dodecyl sulfate- polyacrylamide gel electrophoresis, SHBG = sexhormone binding globulin.

The association between α_1 -antitrypsin (α_1 -AT) and liver disease was noted by Sharp in 1971 (1) with the observation of the α_1 -AT deficiency of phenotype Pi (protease inhibitor) ZZ in patients with juvenile cirrhosis and the demonstration of periodic acid-Schiff (PAS) stainable inclusions after diastase treatment in the hepatocytes. Abnormal frequencies of cirrhosis and hepatoma were later reported among Pi ZZ adults (2). α_1 -AT immunoreactive inclusion bodies from a Pi ZZ liver have been chemically characterized and shown to consist of α_1 -AT with an amino acid substitution (3) in the core-glycosylated (high mannose) state. It has been postulated that the amino acid substitution renders the molecule unstable or insoluble in the endoplasmic reticulum thus preventing transport to the Golgi (1, 3) for complex glycosylation and subsequent export.

Similar inclusion bodies have also been reported in selected patients with phenotype Pi M, high plasma levels of α_1 -AT and active disease (4, 5, 6). In one study of hepatocellular carcinoma (HC) granular α_1 -AT deposits were found in 73% of cases with globular inclusions present in 6% (7). It appears therefore that α_1 -AT inclusions in these cases may reflect abnormal biosynthesis or glycosylation.

Several hepatoma cell lines are known to produce and export α_1 -AT. Glasgow et al (8) recently characterized a biologically active α_1 -AT from the hepatoma line SK-Hep. Gerber et al (9) have shown α_1 -AT synthesis in another cell line PLC/PRF/5, and have demonstrated diastase-resistant PAS-positive globular inclusions with immunoperoxidase staining for α_1 -AT. We have therefore used this latter cell line to study the biosynthesis of human α_1 -AT. Morphological studies have been performed and the export glycoprotein α_1 -AT has been characterized to evaluate the potential value of this cell line as a model of the secretory defect in the Pi ZZ state.

METHODS

Cell Culture. PLC/PRF/5 cells were maintained in culture in

260 ml culture bottles, area 80 cm^2 (Nunc, Denmark) in minimum essential medium with Hanks' salts and 25 mM Hepes buffer, supplemented with a single batch of 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, 1% non-essential amino acids and 0.1 mg/ml gentamicin (all from Gibco, Chemoferm AB, Sweden). This medium will be referred to as M-FCS. Cells were subcultured at least once a week (split ratio 1:4) by a gentle trypsinizing procedure using 0.025% trypsin-EDTA solution (Gibco).

Intracellular aggregates of α_1 -AT. Light microscopy was performed on cells maintained in the M-FCS medium for 3 and 10 days before harvesting by trypsination, centrifugation at 1600 rpm (500 g_{av}), washing in phosphate buffered saline (PBS, Dulbecco's modification, Gibco) and fixation of the pellets in 4% formaldehyde. Pellets were then sectioned by routine histochemical techniques with hematoxylin-eosin and PAS reagent after diastase treatment. Untreated paraffin sections were also stained specifically for α_1 -AT using the immunoperoxidase method exactly as described (4). Cells cultured for 10 days were washed and centrifuged as above, fixed in 2.5% glutaraldehyde and 1% OsO_4 and electron microscopy was performed by Dr J Mebis on ultrathin epon sections.

Experimental Conditions. Cells were washed repeatedly in PBS, or sterile isotonic saline and serum free medium (SFM) was then added. Unless otherwise specified, Williams Medium E (Gibco) was used with the following additives: Bovine serum albumin, free from fatty acids (Sigma) 600 mg/l, human transferrin (Sigma) 50 mg/l, benzyl penicillin (Astra, Sweden) 100 mg/l, streptomycin sulfate (Glaxo) 50 mg/l, monocomponent insulin (Actrapid, NOVO) 0.5 mg/l, dexametasone (Decadron, Merck, Sharpe and Dome) 4 mg/l and L-glutamine (BDH Chemicals, Poole England) 300 mg/l. All cultures with SFM were performed at 37°C in a humidified atmosphere (80%) containing 5% CO_2 . Culture medium was concentrated using an immersible CX-10 ultrafiltration unit (Millipore) after the addition of a protease inhibitor cocktail to give the follow-

ing final concentrations of N-p-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma) 3.75 mg/l, leupeptin (Boehringer Mannheim) 10 mg/l, pepstatin (Boehringer Mannheim) 10 ml/l, and phenylmethylsulfonyl fluoride (PMSF, Aldrich) 0.001 mol/l.

Quantitation of α_1 -AT. α_1 -AT secreted by the hepatoma cells into the SFM was quantitated by electroimmunoassay (10) using dilutions of a plasma pool from 100 healthy blood donors as reference in 1% agarose containing 0.5% monospecific rabbit anti-serum against human α_1 -AT (available at the laboratory).

Regulation of Biosynthesis. After comparable cell growth and α_1 -AT synthesis had been ascertained in multiple culture dishes the following substances were added to fresh SFM: cycloheximide (Sigma) 36 μ mol/l, colchicine (Sigma) 0.1 and 0.2 mmol/l, tunicamycin (Sigma) 1.25 μ g/ml and 2.5 μ g/ml, 17- β -estradiol (Sigma) dissolved in 95% ethanol, 0.2 or 6 μ g/ml with ethanol concentration 0.5%. In one experiment human sex hormone binding globulin (SHBG), generously supplied by Dr. Per Fernlund, Department of Clinical Chemistry at this hospital, was added to a concentration of 1.8 μ mol/l to dishes containing 0, 0.4, 0.8 or 1.2 μ mol/l estrogen.

The possibility of feed-back induction or inhibition was investigated by the addition of purified human plasma α_1 -AT to the medium in concentrations corresponding to four and eight times the daily α_1 -AT secretion (i.e. ca 0.1 and 0.2 μ mol/l). α_1 -AT was then quantitated in the SFM once daily for 6 days in parallel cultures. Corresponding molar amounts of albumin were added to control cultures. Evaporation losses were replaced by sterile-filtered distilled H₂O and sampling aliquots were replaced by equal volumes of SFM.

Biosynthesis of α_1 -AT: 35 S-Methionine Pulse-Labeling. After trypsinization, cells were cultured in 50 mm plastic petri dishes for one day in the M-FCS medium, washed repeatedly in the SFM and cultured to confluence for 2 more days in this medium. Pulse-chase experiments were then performed as described (11). All

media were spun in an Eppendorff centrifuge immediately after interruption of the chase period to prevent contamination from intracellular proteins which possibly could be released upon lysis of aspirated cells. Immunoprecipitation, enzymatic treatment, electrophoresis and fluorography were then performed exactly as described for isolated rat hepatocyte cultures (11).

Characterization of α_1 -AT secreted by PLC/PRF/5 cells. Gel filtration was performed on 250 μ l SFM or an equimolar amount of human Pi M α_1 -AT diluted to 730 μ l with 0.05 M Tris-HCl pH 7.5 containing 0.5 M NaCl on an ACA 44 Ultragel column (LKB, Sweden) 1.0 x 50 cm. Elution was performed with the same buffer flow rate 3.0 ml/h, fraction volume 10 ml. α_1 -AT in elution fractions was quantitated by electroimmunoassay.

The appearance of PLC/PRF/5 α_1 -AT alone and after mixture with increasing amounts of bovine pancreas trypsin (TRTPCK, Worthington) was compared to that of normal and Pi Z α_1 -AT on crossed immunoelectrophoresis (12) using 0.4 or 0.5% of the above antiserum after separation of proteins by agarose electrophoresis (13).

The isoelectric point of the protein was compared to those for α_1 -AT from selected plasma standards by iso-electric focusing after dialysis against a glycine buffer 0.10 mol/l, pH 7.4 at room temperature and cysteine reduction as described (14).

The pattern of glycosylation of PLC/PRF/5 α_1 -AT was studied using crossed immunoelectrophoresis (12) with the first dimension run in a short stretch of 1% agarose, then in a 1% gel containing Concanavalin A (Con-A, Pharmacia) 1 mg/ml at 200 volts for 2 hr essentially according to Wells et al (15). The second dimension was run in a gel containing alpha-methyl mannoside (Sigma) 0.05 mol/l and 0.4% antiserum.

Neuraminidase treatment of export α_1 -AT was performed according to Carlson and Stenflo (11) using SFM- α_1 -AT concentrated to 2.4 g/l or normal serum. Electrophoretic mobility of desialylated α_1 -AT was studied by crossed immunoelectrophoresis.

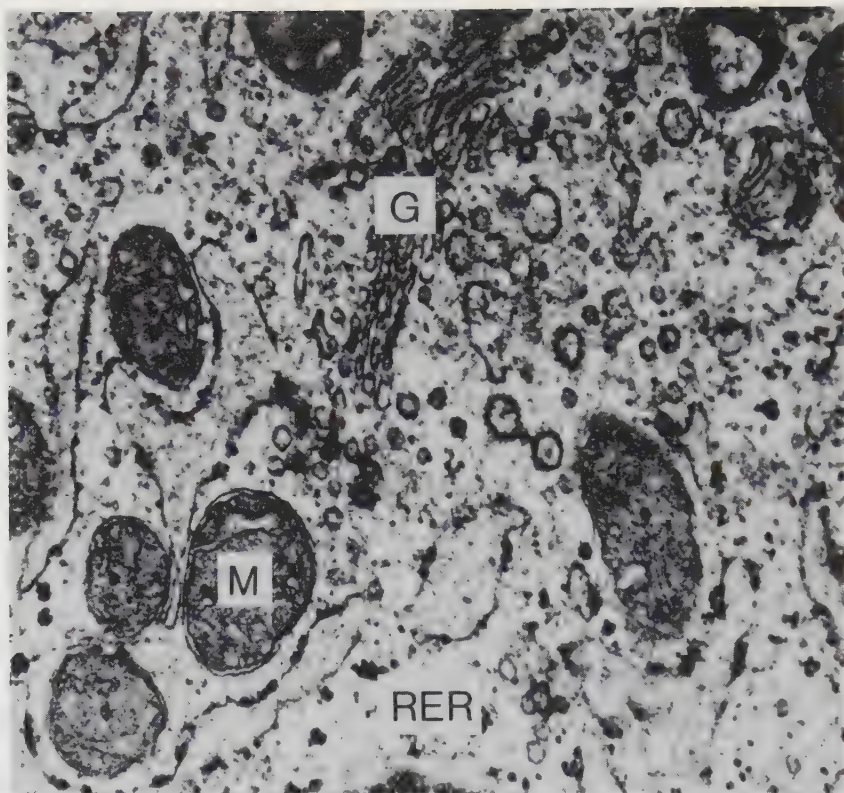


Figure 1. Electron microscopy of a hepatoma cell showing the rough endoplasmic reticulum and Golgi area. The dilated RER contains a granular material; similar material is seen in the lamellae and vesicles of the Golgi. x 58000.

RESULTS

Morphology. Light microscopy of the PLC/PRF/5 cells after PAS-diastase treatment and after immunoperoxidase staining specific for α_1 -AT demonstrated no certain inclusions after 1-2 days culture, granular inclusions in single cells after 3 days and after 10 days incubation in the same medium globular inclusions could be found in half the cells. These results agree with those previously reported by Gerber et al (9). Electron microscopy after 10 days culture demonstrated dilation of the RER but no changes in the cis- or trans Golgi (Fig 1).

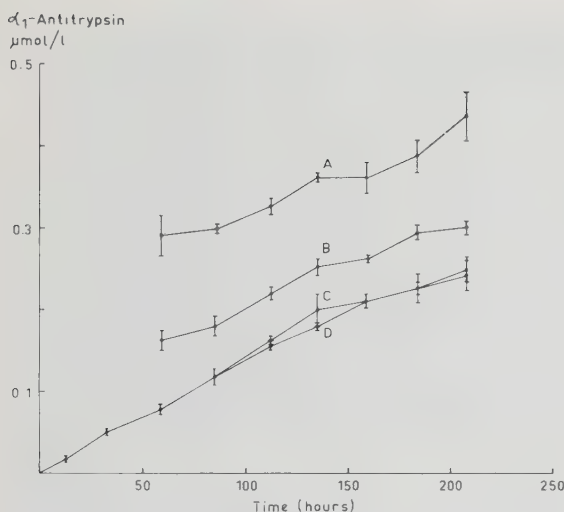


Figure 2. The synthesis rate of α_1 -AT in a feed back experiment. After 2 days incubation additions were made as follows:

A) 0.1 μ mol human α_1 -AT, B) 0.2 μ mol human α_1 -AT, C) 0.1 μ mol human albumin and D) 0.2 μ mol human albumin. Each point is the mean of 5 petri dishes $\pm$ 1 S.D. α_1 -AT addition does not appear to affect the rate of synthesis.

Secretion of α_1 -AT by PLC/PRF/5 cells. Immunoreactive α_1 -AT was clearly demonstrated by electroimmunoassay of both the M-FCS medium and SFM medium after culture of PLC/PRF/5 hepatoma cells. No detectable levels of α_1 -AT could be found in M-FCS or in the SFM prior to culture and no cross reaction was seen on crossed immunoelectrophoresis of the trypsin-FCS antitrypsin complex formed during normal cell transfer. The concentration of α_1 -AT increased successively to about 2.5×10^{-7} mol/l after 10 days in SFM (Fig 2). In the presence of cycloheximide no detectable α_1 -AT was secreted to the medium.

Modulation of α_1 -AT Export. Identical values for the secretion of α_1 -AT were found after incubation of the PLC/PRF/5 cells in the presence of colchicine at concentrations 0, 0.1 and 0.2 mmol/l. Tunicamycin did inhibit the secretion of α_1 -AT during prolonged incubation. After 4 days at a concentration of 2.5 μ g/ml secretion was $32 \pm 1\%$ of controls. 17- β -estradiol, with or without SHBG had no effect on α_1 -AT secretion in the concentrations used. After the addition of purified human α_1 -AT in con-

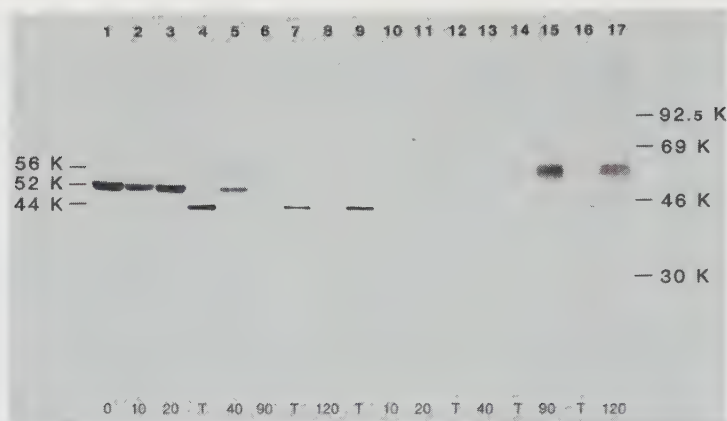


Figure 3. SDS-PAGE of immunoprecipitated α_1 -AT from a pulse-chase experiment. PLC/PRF/5 cells were first incubated for 90 min in methionine free medium with tunicamycin 2.5 μ g/ml in lanes marked T. Pulse time was 10 min 100 μ Ci 35 S-methionine per culture. Immunoprecipitated protein from cell lysates is shown at chase time 0 (lane 1), 10 (lane 2), 20 (lanes 3 and 4), 40 (lane 5), 90 (lanes 6 and 7) and 120 minutes (lanes 8 and 9). Lanes 10 to 17 show immunoprecipitated α_1 -AT from the medium of the cell cultures, where tunicamycin containing cultures are indicated by T. At 40 min chase time a weak band is discerned in the medium of normal cultures and at 90 min the first band was seen in tunicamycin treated cell medium.



Figure 4. Neuraminidase and endo-H treatment of α_1 -AT synthesized in PLC/PRF/5 cells on 10-15% SDS-PAGE with fluorographic enhancement. Aliquots containing α_1 -AT from cell lysates at 0 chase time are shown untreated (lane 1), and after incubation with neuraminidase (lane 2), the endo-H buffer blank (lane 3) and endo-H (lane 4). In lane 5 is seen the α_1 -AT immunoprecipitate from tunicamycin treated cells for comparison. Equal aliquots from 45 min chase time are shown untreated (lane 6), after neuraminidase (lane 7) and endo-H incubation (lane 8). Tunicamycin treated cell lysate in lane 9 for comparison. In lanes 10-12 are shown aliquots of medium untreated (lane 10) after neuraminidase (lane 11) and after endo-H (lane 12). In lane 13 are molecular weight standards for reference.

centrations used no sign of positive or negative feedback could be seen (Fig 2).

Biosynthesis of α_1 -AT. The results of pulse-chase experiments are shown in Figures 3 and 4. The intracellular protein immunoprecipitated with monospecific rabbit antihuman α_1 -AT at 0 chase time had an apparent molecular radius (M_r) of 52,000 daltons on the 10-15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page). After 20 min, a band with M_r of about 56,000 daltons could be discerned and this band increased proportionally in intensity with time. By 40 min a band, indistinguishable from the 56,000 daltons intracellular band became weakly visible in the immunoprecipitate from the SFM. These results agree well with those found for rat α_1 -AT (11). In the hepatoma cell system about 20% of the trichloroacetic acid precipitable radioactivity secreted to the medium at 90 min could be recovered in the α_1 -AT immunoprecipitate. This may be compared to a value of 3.8% for rat α_1 -AT at 90 and 120 min (unpublished).

The identity of the 52,000 dalton band with the core-glycosylated protein containing 3 oligosaccharide side chains was substantiated by digestion with endo- β -N-acetylglucosaminidase (endo-H) (16) as for rat α_1 -AT (11). The band of M_r 56,000 daltons is resistant to digestion by endo-H but alters in appearance after incubation with neuraminidase, verifying that it contains terminal sialic acid residues and corresponds to the complex glycoprotein (Fig 4). In the presence of tunicamycin, a substance known to inhibit the formation of the dolichol-phosphate-oligosaccharide complex in the RER and thus totally prevent glycosylation of Asn-glycosylated glycoproteins (17), a band with M_r of 44,000 daltons was seen. The concentration of tunicamycin was slightly suboptimal in this experiment (2.5 μ g/ml) and a very weak band representing the polypeptide with one oligosaccharide side chain can be seen at about 46,000 daltons in lane 4 (Fig 3). Secretion of α_1 -AT in the presence of tunicamycin was clearly delayed but not totally prohibited and a protein completely lack-

ing in carbohydrate is secreted. These results agree with those previously found for rat α_1 -AT (11, 18).

Characterization of export PLC/PRF/5 α_1 -AT. On gel filtration hepatoma cell derived α_1 -AT appeared at exactly the same elution volume as human plasma Pi M α_1 -AT. Crossed immunoelectrophoresis of the SFM showed a relatively broad α_1 -AT peak with slower mobility than that of Pi M plasma α_1 -AT (Fig 5 a, b). On mixture of constant amounts of SFM with increasing amounts of trypsin, the appearance of a peak cathodal to the original peak demonstrated complex formation and the biological activity of the inhibitor (Fig 6 a, b).

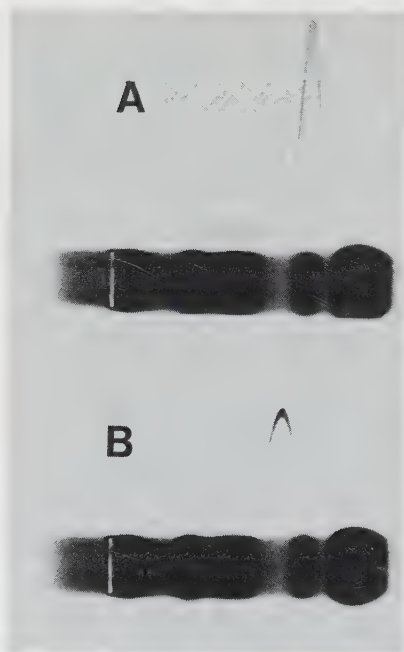


Figure 5. Crossed immunoelectrophoresis at pH 8.6 of α_1 -AT. A) a normal plasma pool and B) serum free medium (SFM) after 3 days culture of PLC/PRF/5 cells. The inset shows the reference agarose electrophoresis for the normal plasma pool. Anti- α_1 -AT 0.5%, anode at right.

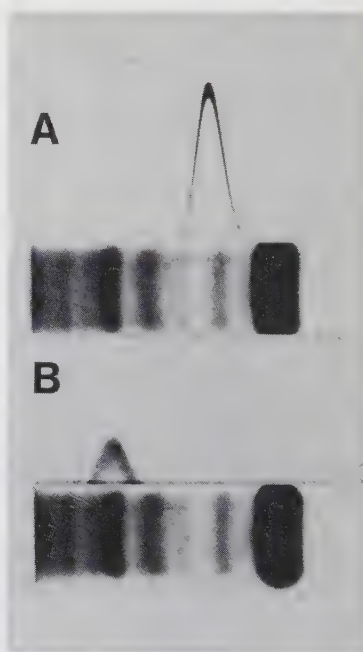


Figure 6. Biological activity of α_1 -AT exported from PLC/PRF/5 cells. Crossed immunoelectrophoresis of SFM after culture of PLC/PRF/5 cells A) untreated and B) in the presence of a molar excess of trypsin, conditions as in figure 5.

Slight variation was seen in repeated runs of isoelectric focusing of the SFM. It was not identical to the patterns for plasma standards of phenotypes Pi M, S, Z or any other known variant (data not shown). On Con A crossed immunoelectrophoresis the pattern for Pi M α_1 -AT contains major bands for isoforms I and II (3 biantennary chains and 2 biantennary, 1 triantennary, respectively) and a weak band for isoform III (1 biantennary and 2 triantennary oligosaccharide chains) (19). These results agree with the normal banding pattern on isoelectric focusing with a weak band 2 (isoform III) and major bands 4 (isoform II) and 6 (isoform I). The pattern for Pi Z differs slightly and that for the SFM significantly from the typical pattern. PLC/PRF/5 contains several varying forms with lower affinity for Con A (higher degree of carbohydrate branching) than Pi M α_1 -AT (Fig 7).

Figure 7. Concanavalin A affinity crossed immunoelectrophoresis of α_1 -AT. Equimolar amounts of α_1 -AT in A) normal plasma pool B) Pi Z, serum plasma and C) SFM after culture of PLC/PRF/5 cells. The approximate positions of isoforms I, II and III are marked. The moderate (A) and large (B) cathodal peaks correspond to non-specific glycoprotein absorption at the Concanavalin A front, during agarose electrophoresis in the first dimension.

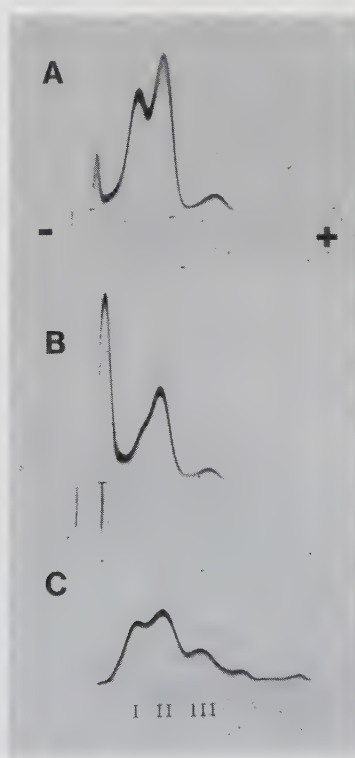


Figure 8. Crossed immunoelectrophoresis of α_1 -AT before and after treatment with neuraminidase. Gels with PLC/PRF/5 α_1 -AT in SFM and normal plasma α_1 -AT are superimposed. The lower peak in each case is SFM. A) Untreated and B) after neuraminidase digestion. Anti- α_1 -AT 0.5%, anode at right.



Neuraminidase treatment resulted in a smaller cathodal shift for PLC/PRF/5 α_1 -AT than for the Pi M protein on crossed immunoelectrophoresis, indicating a lower sialic acid content in the hepatoma protein (Fig 8). Identical results were obtained using the eluates from gel filtration with or without cysteine reduction (14), indicating that the increased width of the PLC/PRF/5 peak is not due to disulfide complex formation.

DISCUSSION

In the PLC/PRF/5 human hepatoma cell line de novo synthesis of immunoreactive α_1 -AT with biological activity was demonstrated and secretion proceeded linearly over a period of 10 days (Fig 2). Synthesis of α_1 -AT by the PLC/PRF/5 cells was totally inhibited by cycloheximide but unaffected by other putative modulating substances. Colchicine in the same concentrations used here has been shown to decrease α_1 -AT secretion from human fetal liver explants (20). It has specifically blocked export of rat α_1 -AT after terminal glycosylation (18) and interrupted the final stages of albumin processing (21). The lack of inhibition in PLC/

PRF/5 cells may indicate altered microtubuli function in the malignant cells.

17- β -Estradiol is known to cause increases in plasma levels of α_1 -AT probably by increasing DNA transcription, and SHBG is thought to mediate this effect (22). In this study 17- β -estradiol had no effect in concentrations greatly exceeding human peak preovulatory values (1.5 nmol/l, ref 22) in either the presence or absence of SHBG. Results indicating initial feed-back induction and subsequent inhibition of α_1 -AT synthesis have been reported after the addition of human α_1 -AT to human fetal liver explants (20) and feed-back induction of prothrombin synthesis has been reported in a rat hepatoma cell line (23). No such regulation could be seen for the biosynthesis of α_1 -AT in the PLC/PRF/5 cells, possibly due to alterations in regulatory mechanisms after malignant transformation of these cells.

The M_r value for the fully glycosylated α_1 -AT secreted in the pulse-chase experiment agrees exactly with the M_r previously reported for human α_1 -AT using SDS-PAGE for M_r determination (24). Considering the intracellular accumulation of α_1 -AT in these cells, the time scale for the biosynthesis and secretion of human α_1 -AT was very similar to those found for rat α_1 -AT (11). The electrophoretic band for the intracellular terminally glycosylated α_1 -AT was broader (Fig 3) than that found for the corresponding rat α_1 -AT. The similar appearance of this band and the export protein indicate heterogeneity in the complex oligosaccharide rather than the presence of intracellular forms with intermediate degrees of glycosylation. The distinctly demarcated bands for the core-glycosylated form and for the polypeptide synthesized in the presence of tunicamycin indicate that the heterogeneity is located in the carbohydrate rather than in the polypeptide moiety of the molecule.

Heterogeneity of the export glycoprotein was further demonstrated by the relatively broad peak seen on crossed immunoelectrophoresis (Fig 5) and verified by the appearance of atypical

anodal peaks with low Con-A affinity (Fig 7). These additional minor peaks should represent forms with a greater degree of carbohydrate branching than isoform III. If completely sialylated, PLC/PRF/5 α_1 -AT would thus have a higher content of terminal sialic acid residues than normal plasma α_1 -AT. The combined results of Figures 5-8 are therefore consistent with incomplete sialylation of a highly branched glycoprotein as were the results of iso-electric focusing. In acute phase reactions a relative increase of α_1 -AT isoform III has been shown (19) but no additional atypical forms have been reported. The presence of abnormal membrane glycoproteins in malignant cells is well known, but to our knowledge only one case report describing incompletely sialylated export glycoproteins in HC has appeared (25).

Globular inclusions of α_1 -AT are normally found in the RER of Pi ZZ hepatocytes. The PLC/PRF/5 cells also have dilated cisternae of the RER (Fig 1) suggesting that the intracellular accumulation occurs at the same level as in Pi ZZ hepatocytes, presumably in the core-glycosylated state. Other cases of globular or even granular inclusions in the absence of the Z allele appear to be associated with high rates of α_1 -AT synthesis and may thus indicate limited access to the mechanisms for terminal or complex-glycosylation. It is interesting to note that the non-glycosylated polypeptide formed in the presence of tunicamycin was secreted, although more slowly than normal α_1 -AT, but no export of the core-glycosylated protein could be demonstrated. Thus the glycosylation process appears to facilitate the intracellular transport and secretion of α_1 -AT whereas terminal glycosylation seems to be a prerequisite for export of the core-glycosylated protein. It is known that carbohydrate attachments may modulate intracellular protein transport (26) and it has been suggested that transport molecules may be present in the RER which can bind with varying affinities to glycoproteins and facilitate transport to the Golgi (27). Thus rapid synthesis might create competition of binding sites with subsequent accumulation in the RER where

minor modification of the core-oligosaccharide may occur. Rapid synthesis may alternatively cause a relative deficiency of carbohydrate substrates or enzymes, e.g. sialic acid or sialyltransferase, explaining the carbohydrate modifications seen in the PLC/PRF/5 export product.

Using the PLC/PRF/5 hepatoma cell line we have delineated the biosynthetic steps for human α_1 -AT. This process is surprisingly similar to that found for rat α_1 -AT (11) with a similar time scale for biosynthesis, post-translational modifications and secretion. Nonetheless, accumulation of α_1 -AT in the RER resembling that seen in the human Pi ZZ occurs. Variations in the carbohydrate structure of exported PLC/PRF/5 α_1 -AT suggest differences in mechanisms of aggregation in these two systems. This cell line is therefore of limited value as an experimental model for the secretory defect in the Pi ZZ deficiency state but may be more suitable for studies of intracellular glycoprotein transport mechanisms. Considering the increased frequency of hepatoma among Pi ZZ individuals (2), we anticipate the application of methods reported here on a human Pi ZZ hepatoma cell line when available.

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Note added in press: Since submission of this manuscript a monoclonal antibody specific for Pi Z α_1 -AT has been used in an ELISA procedure to identify carriers of the Pi Z gene. This antibody does not react with α_1 -AT secreted from PLC/PRF/5 cells.

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